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THYROID CLEARANCE, UPTAKE, AND RATE OF UPTAKE OF RADIOIODINE IN HYPERTHYROIDISM

THEIR INTERRELATIONSHIPS AND DIAGNOSTIC VALUE

ALVIN L. SCHULTZ, M.D., AND LESLIE ZIEVE, M.D.
MINNEAPOLIS, MINN.

SINCE the early investigations[1, 2] almost twenty years ago which demonstrated the effectiveness of radioiodine as an indicator of thyroid function, numerous reports have appeared proposing various techniques for appraising thyroid activity. Many of these have been discarded for practical reasons. The thyroid I^{131} uptake has been the most universally used test, although the optimum time to measure the uptake after a tracer dose has not been agreed upon. The rate of thyroid uptake has been studied but not applied generally as a means of diagnosis.[3] Several investigators[4-6] have claimed the thyroid I^{131} clearance to be the most reliable and sensitive indicator of the state of thyroid function. Because of the diverse views as to the most effective means of diagnosing thyroid dysfunction by radioiodine tracer techniques, clarification is desirable.

The present study is concerned with three measures, thyroid uptake, thyroid clearance, and rate of thyroid uptake, attempting to evaluate their relative merits and interdependence as they are used clinically in the diagnosis of hyperthyroidism.

MATERIALS AND METHODS

Patients.—The euthyroid group consisted of 109 hospital patients without goiter or clinical evidence of thyroid disease. All but 2 were males. The age range was 21 to 66 years.

From the Department of Medicine and Radioisotope Service, Veterans Administration Hospital and University of Minnesota, Minneapolis, Minn.
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The hyperthyroid group consisted of 50 patients with unequivocal clinical hyperthyroidism. Forty-four had diffuse and 6 had nodular goiters. Only 3 were females. The age range was 20 to 66 years.

In addition to the clinical findings, the basal metabolic rate and the serum chemical protein-bound iodine concentration were utilized to support the clinical impression in both groups. None of the subjects were receiving medications known to affect thyroid function, and none had cardiac or renal diseases, or disorders which indirectly might influence the test results.

Measurements.—All the tests to be described were performed simultaneously on each patient in the fasting state following an oral dose of 150 microcuries of I^{131}. *Thyroid uptake* measurements were made ½, 1, 2, 3, and 24 hours after the tracer dose, using a technique described previously.[7] *Thyroid clearances* of I^{131} were calculated for the time periods ½-1, 1-2, and 2-3 hours after administration of the tracer dose. Thyroid clearance was calculated as the ratio of the amount of radioactivity taken up by the gland in a given time interval to the average plasma concentration of radioactivity during that interval. The actual formulation used was

$$TC = \frac{U_2 - U_1}{(P_1 + P_2)/2} \times \frac{1}{t_2 - t_1}$$

where U_1 and U_2 are the percentage thyroid uptake at times t_1 and t_2; $(P_1 + P_2)/2$ is the average plasma I^{131} concentration in percentage of the dose per milliliter during time interval $t_2 - t_1$. This arithmetic average of plasma concentrations is used as an approximation to the true average, $\frac{1}{t_2 - t_1} \int_{t_1}^{t_2} P dt$, derived from the exponential curve of plasma concentration. The true average is slightly overestimated by the arithmetic mean, giving clearances that are correspondingly underestimated. Since with time this discrepancy decreases progressively, the early clearances tend to be underestimated slightly more than those that follow. TC is thyroid clearance expressed as milliliters of plasma cleared of I^{131} per minute. For the calculation of the ½-1 hour clearance the plasma I^{131} concentration at one hour was taken as an approximation to the average concentration during the interval ½-1 hour, $t_2 - t_1$ being taken as 30 instead of 60. The methods used for counting plasma I^{131} have been described previously.[7]

Rate of thyroid uptake was calculated from curves based upon five observed points, ½, 1, 2, 3, and 24 hours after a tracer dose. Only five points were used in plotting the curve to simplify the procedure as it might be used in clinical work. The mean rates of uptake in the present study were very similar to those reported by Luellen, Keating, and co-workers[3] in euthyroid and hyperthyroid subjects, using more points to determine the curve of thyroid uptake.

The rate at which the maximum uptake was approached was calculated from the equation, $U_t = U_m (1 - e^{-kt})$ where U_t is the percentage thyroid uptake at any time t, U_m is the maximum percentage uptake, taken to be the 24-hour value in this study, and k is the rate constant.[3] Though we recognize that the 24-hour uptake is often not the maximum value in hyperthyroid patients, it usually does not deviate greatly from the true maximum. In euthyroid patients the 24-hour uptake is almost invariably higher than any earlier value.

The rate, k, was determined by solving the above equation in its logarithmic form rearranged for convenient calculation, $\log (U_m - U_t) = \log U_m - kt$. This simply represents a plot on semilogarithmic paper of the differences between uptakes during the first 3 hours and the maximum uptake. If one arbitrarily plots in similar fashion the differences between uptakes during the first 3 hours and the dose given, one also gets a straight line whose slope reflects the rate at which the uptake by the gland during this time interval approaches the dose administered. This we have called the rate to dose. Though an arbitrary calculation, it has the merit of a single reference point (the dose given) for both hyperthyroid and euthyroid patients, and as a consequence the measure takes into account differences in final uptake achieved by the two types of patients as well

as the differences in rate of uptake by the gland. As will be seen, it has particular value for discriminating between euthyroid and hyperthyroid patients, even though it has no general meaning in terms of the whole curve of uptake by the gland. These two rates were calculated for each patient, being expressed as the percentage of the maximum or dose taken up per hour during the time interval studied.

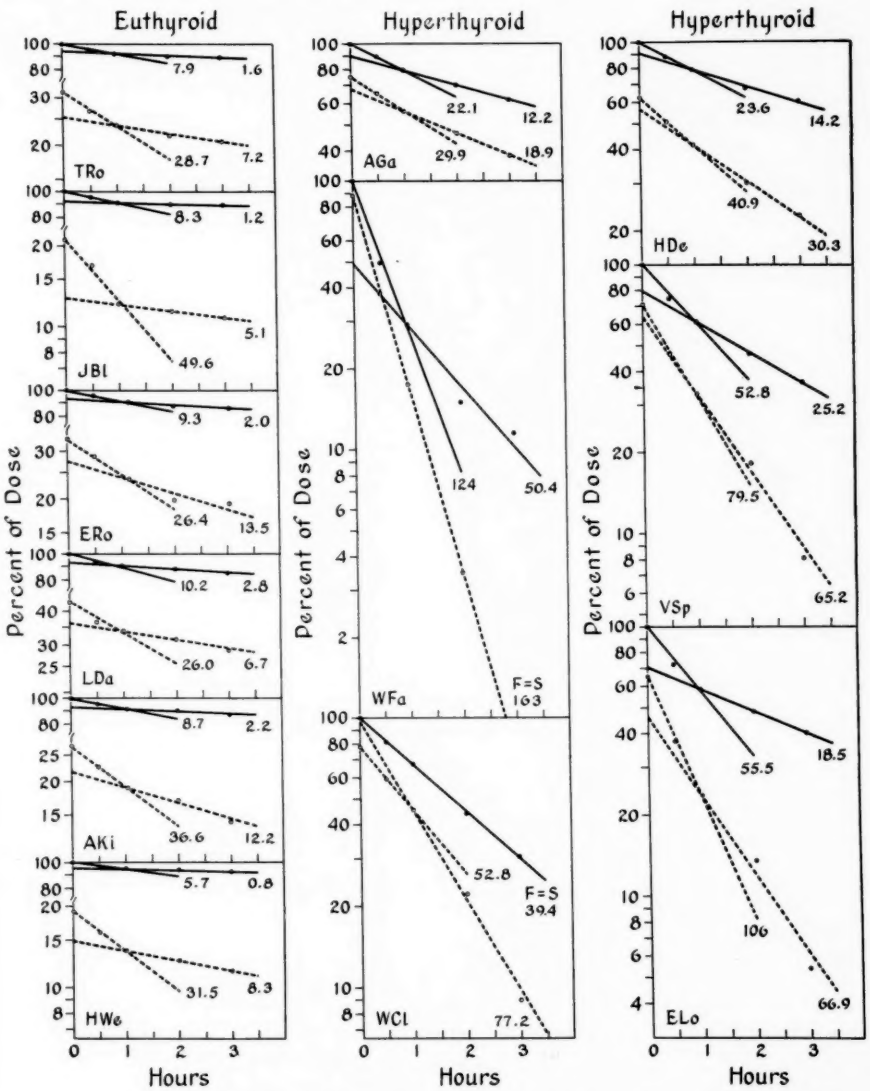

Fig. 1.—Semilogarithmic plots used in calculating rates of uptake of radioiodine in 6 euthyroid and 6 hyperthyroid patients. Solid lines used in calculating rates to dose, broken lines rates to maximum. Number recorded at the end of each line is the slope of the line which is the rate constant in per cent of the dose per hour.

In plotting $(U_m - U_t)$ on semilogarithmic paper it was observed that except in some severely hyperthyroid patients two components were evident. The first, a rapid component, occurred during the first hour and reflected the increasing blood concentration resulting from absorption of the oral dose during this interval. The second slower component reflected the decreasing blood concentration which was apparent after the first hour. Representative examples of the semilogarithmic plots of $(U_m - U_t)$ against time

are given in Fig. 1 for 6 euthyroid and 6 hyperthyroid patients. The two components were evident whether the rate to the maximum (broken lines) or the rate to the dose (solid lines) was plotted. Among the hyperthyroid patients, whose rates of uptake were faster, the change in slope on going from the first to the second component tended to be less than among euthyroids. An occasional severely hyperthyroid patient had an uptake curve adequately represented by a single exponential and did not show two components on the semilogarithmic plot. In such a case the first (fast) and second (slow) components were equal as in the rate to maximum curve of Patient WFa, and the rate to dose curve of Patient WCl. Very rarely a hyperthyroid patient showed a slower first than second component as in the rate to maximum curve of Patient WCl.

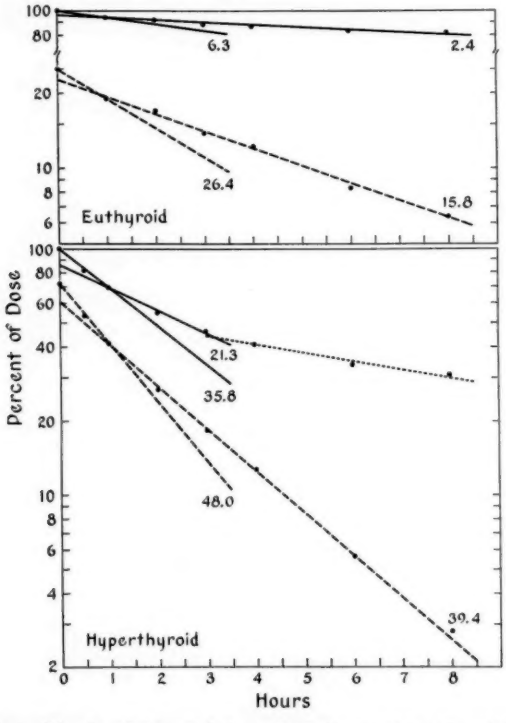

Fig. 2.—Semilogarithmic plots used in calculating rates of uptake of radioiodine for the data summarized in Fig. 4 of the paper of Luellen, Keating, and co-workers.[3] Remainder of legend as in Fig. 1.

These two components of the rate curves are apparent in the data of Luellen, Keating, and associates[3] as shown in Fig. 2 reconstructed from their average curves (assuming, for purposes of plotting, an average 24-hour uptake of 72 per cent for the hyperthyroids and 25 per cent for the euthyroids). A single exponential adequately represents the slow component of the curve among their euthyroid patients during the time interval from 1 to 8 hours whether one plots the rate to maximum or rate to dose. A similar observation is evident for the rate to maximum curve of the hyperthyroid patients but not for the rate to dose curve, which requires a third exponential to account for the segment beyond three hours, as indicated by the dotted line.

Analysis.—Frequency and cumulative frequency distributions were plotted for each variable and each group of patients. In plotting the frequency graphs, the normal range of each test was subdivided into five class intervals. Thus, in terms of class intervals the distance along the abscissa of each graph is identical from test to test for the euthyroid subjects, and the spread of hyperthyroid values may be compared. Means and standard deviations are recorded for each distribution.

Intercorrelations among the measures were calculated for the 50 hyperthyroid patients and for 50 euthyroid subjects selected at random from the larger group.

RESULTS AND INTERPRETATIONS

Thyroid Uptake.—Although there was effective separation of the euthyroid and hyperthyroid groups at all time intervals studied (Fig. 3), the best separation of the two groups occurred at 3 hours. Uptakes within the first

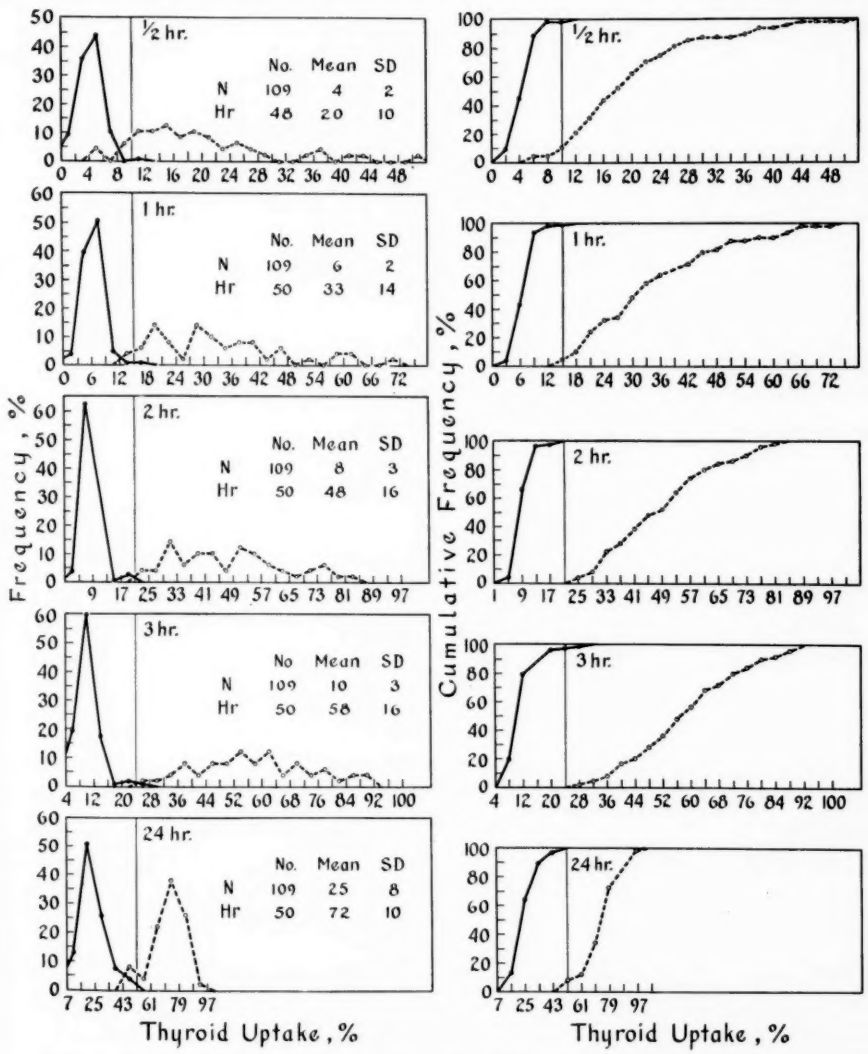

Fig. 3.—Frequency distributions of thyroid uptake of radioiodine at various time intervals, by group. Solid line represents euthyroid or normal (*N*) subjects. Broken line represents hyperthyroid (*Hr*) patients.

3 hours after an oral tracer dose were superior to the 24-hour uptakes, as evidenced by wider separation of the means for the two groups, slightly less overlapping, and less compression of the range of values of the hyperthyroid group. The mean uptakes at 3 hours were 10 ± 3 per cent and 58 ± 16 per

cent for the euthyroid and hyperthyroid groups, respectively. At 24 hours the mean values were 25 ± 8 per cent for the euthyroid group and 72 ± 10 per cent for the hyperthyroid group.

At 3 hours, one-half of the euthyroid subjects had uptake values less than 10 per cent and 96 per cent had values under 20 per cent. One-half of the hyperthyroid group had values exceeding 57 per cent, and 96 per cent exceeding 32 per cent. At 24 hours, 90 per cent of the euthyroid group had uptake values under 35 per cent and only one patient had a value above 50 per cent. Ninety-six per cent of the hyperthyroid group had uptake values above 56 per cent, and only one patient had a value below 50 per cent.

Based upon our sample of 109 euthyroid subjects, we would set the upper limits of normal for thyroid uptake at 1, 2, and 3 hours after a fasting oral dose as 15, 20, and 25 per cent, respectively.

Thyroid Clearance.—Among the hyperthyroid patients, the thyroid I^{131} clearance decreased rapidly in the first 3 hours, as a rule, as indicated by the mean values in Fig. 4. This was also true for the euthyroid group, but less apparent because of the smaller clearances. The percentage decrease in clearance was similar for the two groups. Between the intervals, ½-1 hour and 1-2 hours, there was a decrease in clearance of 45 per cent for the euthyroid group and of 34 per cent for the hyperthyroid group. Between the 1-2 hour and 2-3 hour intervals, the decreases in clearance for the euthyroid and hyperthyroid groups were 10 per cent and 14 per cent, respectively.

Separation of euthyroid and hyperthyroid patients was good at all time intervals studied (Fig. 4). There was slightly more overlapping at ½-1 hour than at later intervals; however, there was little to choose between the 1-2 hour and 2-3 hour periods. The mean thyroid I^{131} clearances at the various time intervals are indicated in Fig. 4.

At 2-3 hours, 50 per cent of the euthyroid I^{131} clearances were below 8 ml./min., 90 per cent were less than 19 ml./min., and 96 per cent were less than 25 ml./min. Fifty per cent of the hyperthyroid I^{131} clearances were above 120 ml./min., 90 per cent were above 42 ml./min., and 96 per cent were above 30 ml./min.

Based upon the sample of 109 euthyroid subjects the upper limits of normal for thyroid I^{131} clearance at ½-1, 1-2, and 2-3 hours may be set at 40, 30, and 25 ml. per minute, respectively.

Rate of Thyroid Uptake.—As one might expect, the rate at which the dose was taken up was superior to the rate at which the maximum uptake was reached, for purposes of discriminating between euthyroid and hyperthyroid patients. Likewise in each instance, the rate for the slow component was more discriminative than the rate for the rapid component. Thus the most effective separation of groups was obtained with the slow component of the rate at which the dose was accumulated (Fig. 5). In severely hyperthyroid subjects, however, the distinction between rapid and slow components occasionally could not be made. The types of rate of uptake curves encountered have been illustrated in Fig. 1.

Analysis of the slow component of the rate to dose revealed that 50 per cent of the euthyroid group had rates below 2.2 per cent/hour, and 96 per cent had rates below 4.8 per cent/hour; whereas 50 per cent of the hyperthyroid group had rates above 25.2 per cent/hour, 96 per cent had rates above 8.4 per cent/hour, and 100 per cent had rates above 6.0 per cent/hour.

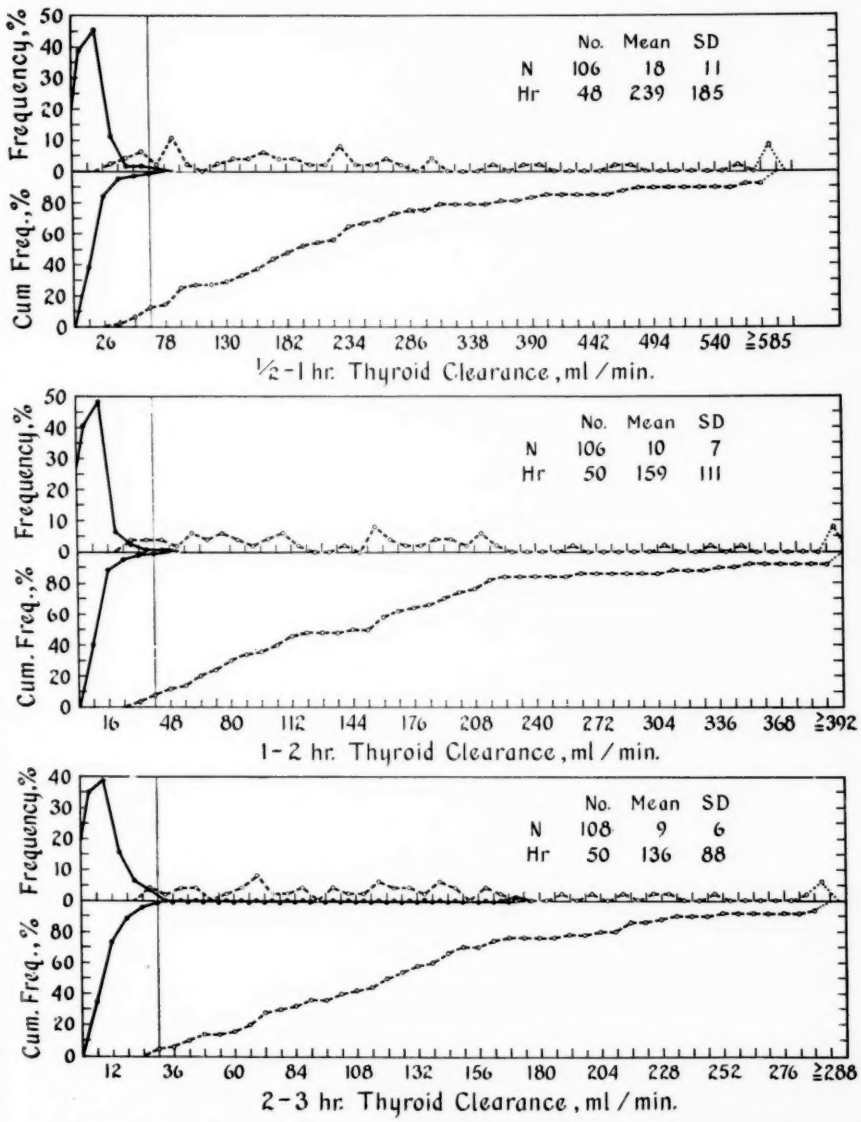

Fig. 4.—Frequency distributions of thyroid clearance of radioiodine at various time intervals, by group. Top half of each segment represents frequency distributions; bottom half, cumulative frequency distributions. Remainder of legend as in Fig. 3.

Interrelationships.—Intercorrelations were calculated among the measures as shown in Table I. Scatter diagrams for the hyperthyroid patients are presented in Fig. 6. For the euthyroid as well as the hyperthyroid patients, the

correlations among the measures were in general moderately high. The best correlation observed among the hyperthyroid patients was between the 3-hour thyroid uptake and the slow component of the rate to dose (0.93).

When both euthyroid and hyperthyroid patients were analyzed together, the intercorrelations rose, as would be expected, ranging in value from 0.77 for the 24-hour uptake and the 2-3 hour I^{131} clearance to 0.96 for the 3-hour uptake and the slow component of the rate to dose. The 3-hour uptake was

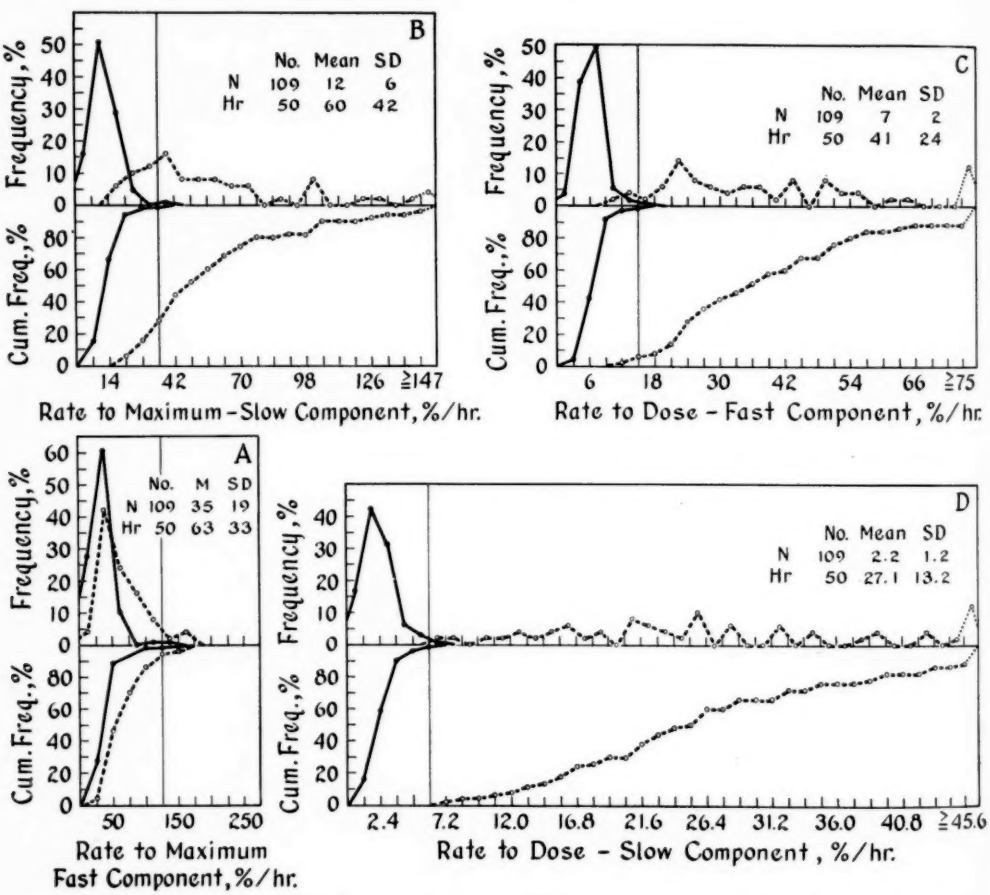
B
No. Mean SD
N 109 12 6
Hr 50 60 42
Frequency, %
Cum. Freq., %
14 42 70 98 126 ≥147
Rate to Maximum - Slow Component, %/hr.
C
No. Mean SD
N 109 7 2
Hr 50 41 24
6 18 30 42 54 66 ≥75
Rate to Dose - Fast Component, %/hr.
A
No. M SD
N 109 35 19
Hr 50 63 33
50 150 250
Rate to Maximum Fast Component, %/hr.
D
No. Mean SD
N 109 2.2 1.2
Hr 50 27.1 13.2
2.4 7.2 12.0 16.8 21.6 26.4 31.2 36.0 40.8 ≥45.6
Rate to Dose - Slow Component, %/hr.

Fig. 5.—Frequency distributions of rates of thyroid uptake of radioiodine, by component and group. Remainder of legend as in Fig. 4.

more highly correlated with the other two measures than was the 24-hour uptake. The 3-hour uptake and the 2-3 hour I^{131} clearance, rather than values at earlier time intervals, were selected for these correlation studies, because of their slight superiority in separating the euthyroid and hyperthyroid groups. The correlation between the 3-hour uptake and the ½-1 hour thyroid I^{131} clearance in hyperthyroid patients was 0.75, slightly higher than the value of 0.64 obtained when the 2-3 hour clearance was used. The correlation between the ½-1 hour and 2-3 hour clearances was 0.70.

3 hr. Thyroid Uptake, %
r = 0.93
Rate to Dose – Slow Component, %/hr.
3 hr. Thyroid Uptake, %
r = 0.64
2–3 hr. Thyroid Clearance, ml /min.
3 hr. Thyroid Uptake, %
r = 0.56
24 hr. Thyroid Uptake, %
r = 0.56
Rate to Dose – Slow Component, %/hr.
24 hr. Thyroid Uptake, %
r = 0.44
2–3 hr. Thyroid Clearance, ml /min.
Rate to Dose – Slow Component, %/hr.
r = 0.64
2–3 hr. Thyroid Clearance, ml /min.

Fig. 6.—Scatter diagrams and intercorrelations among tests within the hyperthyroid range.

The 3-hour thyroid uptake was clearly as effective as the other measures in separating the euthyroid and hyperthyroid groups as shown in Figs. 3, 4, and 5. In view of this and the high intercorrelations of the 3-hour uptake with the rate of uptake and the thyroid I^{131} clearance, this simplest of measures is probably adequately representative of the others for practical clinical purposes. On the same bases, it is apparent that the 24-hour uptake is somewhat less effective and less representative.

TABLE I. INTERCORRELATIONS AMONG TESTS

	24 HR. TU	RTU-S	2-3 HR. TC
3 hr. TU			
Euthyroids	0.68	0.76	0.74
Hyperthyroids	0.56	0.93	0.64
Both	0.93	0.96	0.84
24 hr. TU			
Euthyroids		0.61	0.47
Hyperthyroids		0.56	0.44
Both		0.86	0.77
RTU-S			
Euthyroids			0.60
Hyperthyroids			0.64
Both			0.84

Number of cases: euthyroids 50, hyperthyroids 50, both 100.
TU = Thyroid uptake.
TC = Thyroid clearance.
RTU-S = Rate of thyroid uptake of dose, slow component.

DISCUSSION

Previous reports have shown that the thyroid I^{131} uptake will distinguish hyperthyroid from euthyroid subjects within the first hour following a tracer dose.[8-10] Separation was considered as good as that obtained with the 24-hour thyroid uptake. Results were similar whether an oral or an intravenous dose was given.[9]

We found the uptake at 3 hours to be the best uptake measurement after an oral tracer dose, although it had little advantage over the 1- and 2-hour time intervals, and there was good separation of groups as early as ½ hour after the dose. The 24-hour uptake was less effective, judging by the degree of separation of the means of the groups, the amount of overlapping, and the spread of hyperthyroid values. This is not surprising when one considers the shape of the uptake curve in hyperthyroid patients in whom, in contrast to euthyroid subjects, the major accumulation of I^{131} by the thyroid occurs within the first 8 hours. Also, in the more severely hyperthyroid patients there is a significant decline in thyroid radioactivity during the second 12-hour period as a result of the release of labeled hormone. By the same token one would expect uptake values up to 8 hours to be as discriminative as the 3-hour value.[11]

It has been claimed that thyroid clearance of I^{131} remains constant for several hours after the dose is given.[5, 6, 12] Our observations indicated a progressive decrease in the I^{131} clearance during the first 3 hours, as a rule. This was more apparent in hyperthyroid patients than in euthyroid subjects whose initial clearance values are small. Though the absolute decrease in clearance

in euthyroid patients might be ignored as small, the proportionate decrease is similar, on the average, to that in hyperthyroid patients. The lack of constancy of radioiodine clearance is best studied in the hyperthyroid patient whose values are sufficiently large to reveal striking absolute changes.

In the study by Myant, Pochin, and Goldie[5] observations on 2 hyperthyroid patients revealed a progressive decrease in thyroid I^{131} clearance with time, though the authors concluded the clearance remained constant. Berson and associates[6, 12, 13] have emphasized the constancy of thyroid I^{131} clearance over periods of several hours and have utilized this assumption in calculations of the rate of thyroidal iodide binding and the rate of hormone formation. Their data consisted of (a) 4 euthyroid subjects each of whom showed a slight decrease in clearance upon comparison of the first ½ hour with the last 2½ hours of a 3-hour period,[6] and (b) 3 hyperthyroid patients each of whom showed *nonrandom* changes (first increasing then decreasing) in thyroid I^{131} clearance during the brief periods they were studied.[12] The sequence of values clearly reflected the differences in the rate of change of thyroid uptake as compared to the rate of change in plasma concentration. These data seem to us inadequate support for the conclusions drawn by the authors. They have furthermore implied that the thyroid I^{131} clearance may only be constant for very short periods after the dose because of the rapid appearance of significant concentrations of PBI^{131} in the plasma, particularly in very active glands.

TABLE II. PERCENTAGE OF PLASMA RADIOACTIVITY THAT IS PROTEIN-BOUND AT VARIOUS TIME INTERVALS AFTER THE DOSE IN REPRESENTATIVE HYPERTHYROID, EUTHYROID, AND HYPOTHYROID PATIENTS

PATIENTS	DOSE	PLASMA (PBI^{131}/TOTAL I^{131}) × 100						THYROID UPTAKE	
		½ HR. (%)	1 HR. (%)	2 HR. (%)	3 HR. (%)	5 HR. (%)	24 HR. (%)	3 HR. (%)	24 HR. (%)
Hyperthyroid									
RCl	Oral	0.7	1.0	0.8	1.6	--	62.9	35.6	61.0
EBl	Oral	--	1.2	1.6	3.1	9.9	86.7	53.0	70.6
ETo	Oral	--	0.9	2.8	7.7	32.5	76.2	69.8	73.5
VSp	Oral	--	2.1	5.2	11.4	37.2	81.2	63.7	71.8
SBu	I. V.	1.2	1.7	1.3	1.6	--	25.5	26.3	48.2
Euthyroid									
RRe	Oral	--	1.6	1.5	1.3	2.3	3.1	8.8	19.9
GWo	Oral	--	1.3	1.4	1.2	1.5	4.3	12.2	31.5
HLo	Oral	--	1.5	1.6	1.7	1.5	6.1	9.7	22.2
AMi	Oral	--	2.5	2.6	2.3	2.2	4.8	7.5	19.4
JGu	I. V.	1.1	1.3	1.4	1.5	--	6.0	12.5	29.3
Hypothyroid									
OJo	Oral	--	0.3	0.3	0.3	0.6	2.8	4.0	3.5
NWi	Oral	--	0.1	0.2	0.3	0.3	1.1	4.4	3.9
AWe	Oral	--	0.8	1.0	1.5	2.6	8.6	10.0	15.9
JKe	Oral	--	0.3	0.2	0.4	0.4	1.4	4.1	2.1
HSa	Oral	--	1.9	1.6	1.6	1.9	4.6	4.7	7.1

This presumably is their explanation for the rapid decrease in clearance most evident in severely hyperthyroid patients beyond the first half hour. We have tested this thesis by measuring plasma PBI^{131} concentrations ½, 1, 2, 3, 5, and 24 hours after the dose[14] and calculating conversion ratios as shown in Table II. Little difference is noted between the euthyroid and hyperthyroid patients within the first two hours. Even in severely hyperthyroid patients

(ETo and VSp) the PBI^{131} is only a small fraction of the circulating radioiodine at 3 hours and so could only have a negligible effect in the computation of the thyroid clearance. The appearance of significant amounts of labeled hormone in the blood is not as rapid as presupposed by these workers, and the concentration of labeled hormone is apparently not a significant factor in the progressive decrease in thyroid I^{131} clearance with time during the early hours after a dose.

The decreasing I^{131} clearance most likely reflects the changing distribution of radioiodine. The intravascular-extravascular distribution of a test substance influences any clearance method which uses a single dose technique, as has been discussed by Smith[15] in relation to renal clearances. An essential simplifying condition is constancy of the blood level. This does not exist after a single injection because of the rapid flow of the substance from the intravascular to the extravascular compartments, equilibrium not being reached for many hours. Though one might ignore slight losses elsewhere, as through renal excretion, one cannot ignore the significant fraction flowing into the extravascular nonthyroidal space during the early hours when a clearance calculation is feasible. For the thyroid I^{131} clearance to be constant under these conditions, the rate of change in plasma I^{131} concentration must be proportional to the rate of change in gland uptake of I^{131}. This is an unlikely event under the circumstances and is not borne out by our data.

The true thyroidal clearance of iodine (I^{127}) is probably fairly constant over a given interval of time. However, it is not reflected accurately in clearances of I^{131}, although at some one variable time after the tracer dose the true clearance may be approximated. Nevertheless, I^{131} clearance studies, such as have been presented, will distinguish hyperthyroid from euthyroid individuals and have practical diagnostic value.

Little clinical application has been made of the rate of accumulation of I^{131} by the thyroid gland. Luellen, Keating, and co-workers,[3] studying small groups, observed broad separation of hyperthyroid and euthyroid patients. Their data, like ours, showed a rapid and a slow component in the rate of uptake after an oral dose. The rapid component is probably related to absorptive factors, reflecting the rapid rise in plasma I^{131} which reaches a peak approximately one hour after a fasting oral dose, and only indirectly reflecting the thyroid functional state. The slow component represents the diminishing increments of thyroid uptake as the plasma concentration decreases gradually. It reflects the thyroid functional state directly and was more effective than the rapid component in separating the hyperthyroid and euthyroid groups. Also, the rate at which the dose was taken up during the first 3 hours was more discriminative than the rate at which the maximum uptake was approached. This is to be expected since the ultimate level of uptake achieved is taken into account as well as the actual rate of uptake.

The rate of thyroid uptake was at least as effective as the thyroid I^{131} clearance in differentiating hyperthyroid from euthyroid patients. However, neither of these measurements was any more discriminative than the simpler 3-hour uptake. All three measures reflect the iodine-accumulating function

of the thyroid gland, and their intercorrelations were in fact high when the measurements were made simultaneously in the same patients. In view of these observations the 3-hour uptake as the simplest measure becomes the clinical procedure of choice, and little additional information is gained by adding the other two tests.

SUMMARY

Tests were performed simultaneously in 109 euthyroid and 50 hyperthyroid patients. Plasma thyroid clearances were obtained ½-1, 1-2, and 2-3 hours after an oral dose of I^{131}. A progressive decrease in I^{131} clearance was the rule. Clearances at each time interval were of equal value in separating hyperthyroid from euthyroid patients. The rates at which the dose was taken up and the maximum reached were also calculated. Two components, the first fast, the second slower, were evident in the rate curves. The slow components were more discriminative than the fast, and the rate to dose was more discriminative than the rate to maximum. Uptakes at ½, 1, 2, and 3 hours each separated the groups effectively, the 2- and 3-hour measurements being best and equivalent in value to the clearance and the dose rate. The 24-hour uptake was less discriminative. The 3-hour uptake, rate to dose, and thyroid clearance were highly interdependent. Correlations among these three measurements were: first and second 0.96, first and third 0.85, and second and third 0.84. The 3-hour uptake was representative of and as effective as the more complicated measures.

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THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

V. The Effect of Supplementary Sulfur-Containing Dietary Amino Acids Upon the Die-Away Plots of Autosynthesized Albumins and Gamma Globulins Labeled With S^{35}

David Bronsky, M.D., Samuel Hyman, M.D., and
S. Howard Armstrong, Jr., M.D.
Chicago, Ill.

INTRODUCTION

INVESTIGATION of the apparent turnover times of plasma protein fractions autosynthesized following oral S^{35} has failed to develop, either in our studies[1-3] or in those of Volwiler and his associates,[4] a method of measuring protein degradation rates acceptable for extensive clinical investigation. Autosynthesized protein fractions have usually shown longer apparent turnover times than have comparable intravenously injected in vitro I^{131} or in vivo S^{35}-labeled fractions. These discrepancies have often been attributed to reutilization (recycling) of the isotope tag following its liberation during catabolism of labeled body proteins.[5-7] Luck recently has written: "It is common practice to minimize this source of error by the administration of relatively large quantities of unlabeled cysteine, methionine, or such other amino acids, which by entry into the amino acid pool will dilute out the labeled amino acid released from the protein during metabolism. How effective this device is cannot be determined."[8]

In this study, we have (a) investigated the effect of feeding unlabeled sulfur containing amino acid supplements upon the die-away plots of autosynthesized S^{35}-labeled protein fractions; (b) appraised the suggestion of Volwiler's group[4] that cysteine, since it enters less into the total body organic sulfur pool than methionine, will thus be less subject to reutilization; and (c) compared the intravenous to the oral route for the administration of labeled amino acids.

MATERIALS AND METHODS

Choice of Subjects.—A list of subjects, their diagnoses, and their plasma protein levels are given in Table I.

From the Hektoen Institute of Medical Research of the Cook County Hospital and the Departments of Medicine of the University of Illinois College of Medicine and Northwestern University Medical School.

Presented in part April 19, 1956, at a symposium in Atlantic City sponsored by the Metabolism and Nutrition Study Section of the National Institutes of Health.

These studies have been supported by Grant H-1511 (C, C2) from the National Heart Institute of the United States Public Health Service.

We are indebted to Warden Fred Hertwig of the Cook County Hospital and to the Drs. Leonard H. and Louis D. Weissman Medical Research Foundation for the provision of basic facilities for protein research at the Hektoen Institute.

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Two subjects (J. M. and R. S.) were young adult men with no clinical evidence of abnormal protein metabolism.

Seven additional subjects were chosen to exemplify a range of hypoalbuminemia and represent a variety of nutritional problems. In three, the hypoalbuminemia was severe (T. C., W. S., and T. N.): the remainder showed serum albumin levels ranging up to 3.3 Gm. per 100 c.c.

TABLE I*

PATIENT	DIAGNOSIS	SERUM PROTEINS (GM./100 C.C.)		
		TOTAL	ALBUMINS	GLOBULINS
T. N.	Chronic pancreatitis (autopsy)	4.0	0.7	3.3
Al. J.	Surgical pancreatectomy	7.1	2.8	4.3
Au. J.	Chronic calcific pancreatitis	6.5	3.4	3.1
M. L.	Chronic calcific pancreatitis	6.2	2.9	3.3
L. D.	Idiopathic adult sprue	5.5	2.4	3.1
W. S.	Hypoproteinemia, nutritional (defective surgical anastomosis)	3.0	0.8	2.2
T. C.	Hypoproteinemia, idiopathic, possibly nutritional (senility)	3.7	1.3	2.4
J. M.	Nutritionally normal (cerebral palsy)	7.8	3.9	3.9
R. S.	Nutritionally normal (transverse myelitis)	7.9	4.0	3.9

*A/G ratio by paper electrophoresis as described in reference 1.

TABLE II*

PATIENT	LABEL			ORAL SUPPLEMENT		FIGURE IN THIS PAPER
	ROUTE	DOSE (MC.)	TYPE	DOSE	TIMING AFTER LABEL ADMINISTRATION	
T. N.	Oral	0.5	S^{35} methionine	None		1
Al. J.	Oral	0.5	S^{35} methionine	None		1
Au. J.	Oral	0.5	S^{35} methionine	None		1
M. L.	Oral	0.5	S^{35} methionine	None		1
L. D.	Oral	0.5	S^{35} methionine	None		1
W. S. a.	Oral	0.5	S^{35} methionine	None		2
b.	Oral	0.5	S^{35} methionine	None		2
c.	I.V.	0.1	I^{131} albumin	Lugol's solution		2
d.	I.V.	0.1	I^{131} α-globulins	Lugol's solution		2
T. C. a.	Oral	0.5	S^{35} L-cysteine	5 Gm. cysteine 5 Gm. methionine	Day 108 to day 124	3 and 5
b.	I.V.	0.1	I^{131} albumin	Lugol's solution		3
c.	I.V.	0.1	I^{131} α-globulins	Lugol's solution		3
J. M. a.	Oral	3.0†	S^{35} yeast	5 Gm. cysteine 5 Gm. methionine	Day 275 to day 292	4
b.	Oral	0.5	S^{35} cysteine	Same	Day 18 to day 42	4
c.	I.V.	0.5	S^{35} cysteine	Same	Day 0 to day 21	6
d.	I.V.	0.5	S^{35} cysteine	Same	Day 0 to day 14	8
R. S. a.	I.V.	0.5	S^{35} cysteine	5 Gm. cysteine 5 Gm. methionine	Day 0 to day 14	7
b.	I.V.	0.5	S^{35} cysteine	Same	Same	9

*Protein fractions labeled with I^{131} for intravenous injection were obtained through Dr. Donalee Tabern of Abbott Laboratories; North Chicago, Illinois. These fractions were not especially iodinated for this metabolic study.

†The reasons for the high dosage are given, for this special instance, in reference 3.

Administration of Labels and Supplements: Radioactivity Measurements.—A list of the labeling and supplementation experiments, detailing label, route, and timing, is given for these subjects in Table II, together with a list of corresponding data plots by figure number.

Except when noted in legends, oral and intravenous S^{35} dosage level approximated 0.5 mc.

Supplementary sulfur-containing dietary amino acids were administered orally as methionine and cysteine, 5 Gm. each, daily in divided doses for periods of 14 to 21 days. This is, in our experience, the maximum dose which can be tolerated without producing loathing or gastrointestinal disturbances. Subjects were maintained on the regular hospital diet without the controls usual on a metabolic ward.

Serial sera were withdrawn for periods up to 50 days. In the two instances, where the studies were carried out 275 and 110 days after labeling, residual radioactivity was too low to permit measurement of electrophoretically separated protein fractions, and, therefore, unfractionated serum protein samples were substituted. In all other studies, methods of fractionation, extraction, measurement of component concentrations, and radioactivity were carried out as previously described,[1] and die-away plots were made of the resultant data in the customary semilogarithmic fashion against time.

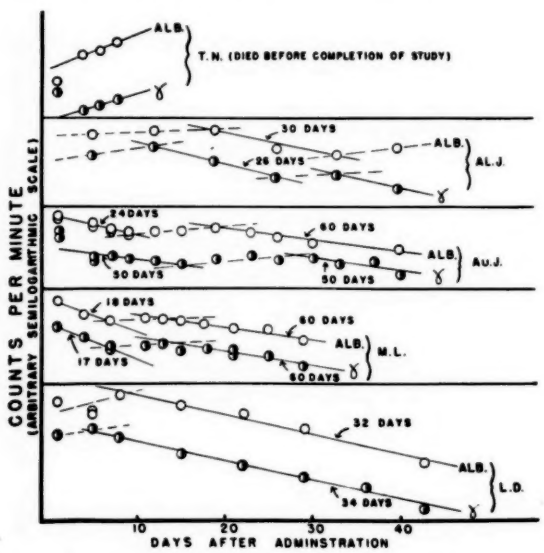

Fig. 1.—Albumin and gamma globulin die-away plots in 5 patients with abnormal gastrointestinal tracts following methionine S^{35} ingestion (see Tables I and II).

Apparent turnover times corresponding to slopes of selected segments are indicated next to the arrows.

RESULTS

Die-Away Plots Following Oral S^{35} Methionine Without Unlabeled S Amino Acid Supplementation.—Customarily, the slope of the first 5 days of a die-away plot is considered chiefly representative of equilibration of the intravascular labeled plasma protein with the extravascular pool. The slope for the next 10 days or so is considered chiefly characteristic of the rate of metabolic degradation of the labeled protein.

In our previously published studies, we have reported a very frequent flattening of the slope of the die-away plots which may begin late in this "metabolic period" and, particularly following oral S^{35}-labeled yeast, may become quite striking between the twentieth and fortieth days. More rarely have we noticed such flattening with an intravenously injected in vitro labeled protein.

In Fig. 1 are presented albumin and gamma globulin die-away plots of the first 5 subjects of Tables I and II. Diagnostic classification is less important

here than the form of the die-away plots, particularly in the first 5 days representative of "equilibration" when the slope should, in the absence of isotope reutilization, be sharply downward. The two top and the bottom subjects have *rising* specific activity levels for albumins and gamma globulins during these first days. The two middle patients in Fig. 1 have specific activity levels which, although showing an initial decline in the mixing and metabolic periods, give some evidence of a rising level before a final falling slope. Whereas the top two patients could not be described as clinically in the "steady state," the bottom three could so be described. Therefore, these roller-coaster die-away plots clearly preclude any graphic translation of slopes into apparent turnover times. The initially encountered rising slopes suggest a label recycling so great as to obliterate completely the downward slope ordinarily characteristic of the equilibration period. We thus concur in the suggestion of Volwiler's group that the oral S^{35} methionine label is not adaptable to clinical studies of plasma protein fraction turnover by die-away plot.

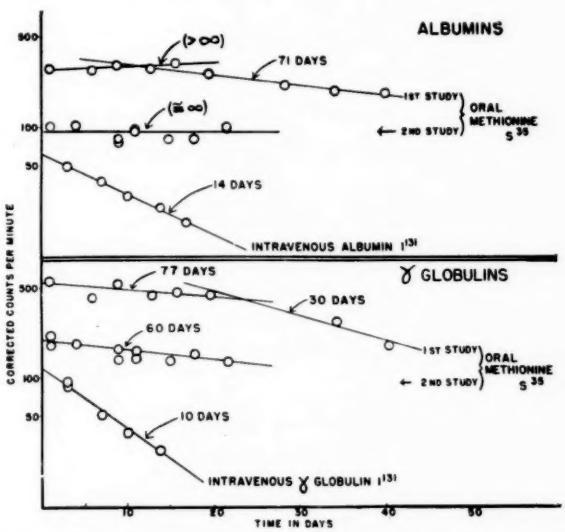

Fig. 2.—Patient W. S. Comparison of the die-away plots of albumins and gamma globulins following oral S^{35} methionine and following intravenous I^{131}-labeled protein fractions. The top two lines in the upper and lower diagrams represent duplicate studies with the oral isotope and show in the first study terminal dropping off rather than a terminal flattening of the die-away plot. A glance shows the great difference between the I^{131} and S^{35} slopes in this subject. Corresponding apparent turnover times are indicated as in Fig. 1.

Comparison in the Same Hypoalbuminemic Subject of the S^{35} Methionine Oral Label With the I^{131} Intravenous Label of Albumins and Gamma Globulins.—Fig. 2 presents three sequential studies of subject W. S. (Tables I and II). It will be noted that in both studies following oral labeling with S^{35} methionine performed without use of supplementation (a) the albumin die-away plots showed either unchanging or increasing specific activity for almost 3 weeks; and (b) the companion die-away plots for gamma globulins, while not rising or flat, show a very slow decrease with time. In contrast, the slope of the plots following intravenous I^{131}-labeled proteins showed a steep decline. This corresponds for albumins to an apparent turnover time 3 or 4 days shorter than

is usually observed in normal subjects. The apparent turnover time for gamma globulins is also somewhat shorter than in the normal. The findings in this subject represent the most accentuated discrepancy between the I^{131} and S^{35} label that we have encountered and confirms our dissatisfaction with oral methionine as a valid label for clinical investigation.

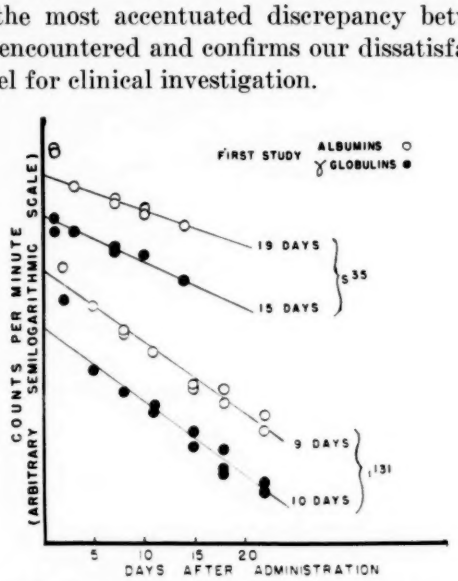

Fig. 3.—Patient T. C. Oral L-cysteine and I.V. I^{131}. Die-away plots on a patient with nutritional hypoalbuminemia secondary to prolonged low protein intake.

The upper two plots are for albumins and gamma globulins following oral S^{35} L-cysteine. The lower two plots are following the intravenous injection of I^{131}-labeled fractions.

Apparent turnover times corresponding to the slopes during the "metabolic decay period" between day 5 and day 14 are indicated to the right of the plots.

Comparison in a Hypoalbuminemic Subject of the S^{35} Cysteine Oral Label With the I^{131} Intravenous Label for Albumins and Gamma Globulins.—Fig. 3 illustrates another nutritional hypoalbuminemic patient (T. C.); here is a comparison between I^{131} and S^{35} apparent turnover times where the oral label was S^{35} L-cysteine and where supplementation was not employed. Here the slope, during the metabolic period, yields apparent turnover times which are significantly shorter than are usually found after the oral S^{35} methionine label, but are still considerably longer than with comparable findings with the I^{131} intravenous label.

This result substantiates the suggestion of Volwiler and his associates that cysteine may be preferable as a labeling agent to methionine from a recycling standpoint. But while lessening, cysteine clearly does not eliminate the discrepancy between the apparent turnover time obtained by the S^{35} and I^{131} method in the same subject.

Comparison of the Effect of Dietary Unlabeled S Amino Acid Supplements on the Apparent Turnover Times of Plasma Proteins at Various Intervals After Oral and Intravenous Administration of the S^{35} Label.—

Unfractionated serum proteins months after oral labeling with S^{35} methionine: Supplementary dietary sulfur containing amino acids were fed to one normal (Fig. 4, top) and one hypoproteinemic (Fig. 5) patient, 275 and 110 days, respectively, after the oral S^{35} label. In each patient, there occurred an immediate steepening of the slope corresponding to an apparent turnover time of 17

days. Compared to the mean of the apparent turnover time of albumins and gamma globulins as measured by intravenously injected I^{131}-labeled fractions, this apparent turnover time was 4 days longer for the normal patient (J. M.) and 7½ days longer for the hypoproteinemic patient (T. C.). In the same manner, the mean apparent turnover time after oral S^{35} L-cysteine in the hypoproteinemic patient was also 17 days. Thus, while supplementary feedings secured a marked increase in apparent turnover time, a discrepancy between the S^{35} and intravenous I^{131} label is still apparent.

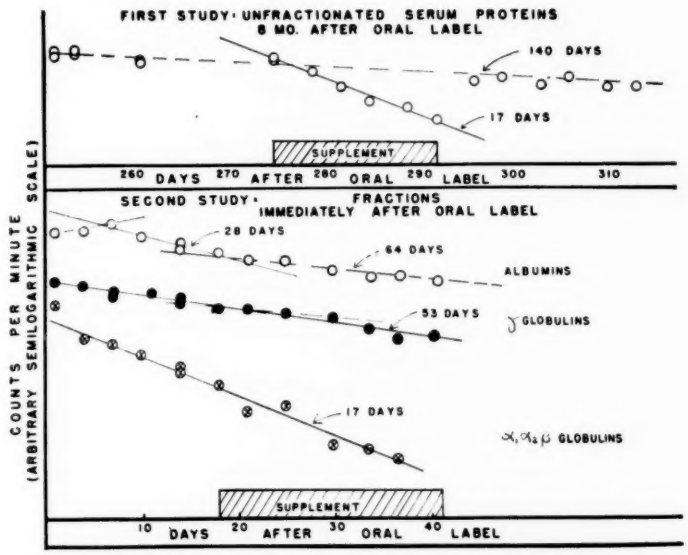

Fig. 4.—Patient J. M. Comparison of effect on die-away plots of oral unlabeled S amino acid supplementation started 275 days (top section) and 18 days (bottom section) after the oral dose of S^{35} amino acid.

Because of low specific radioactivities, fractionation in the first study (upper) was impractical. Fractions prepared as described elsewhere are plotted for the second (lower) study.

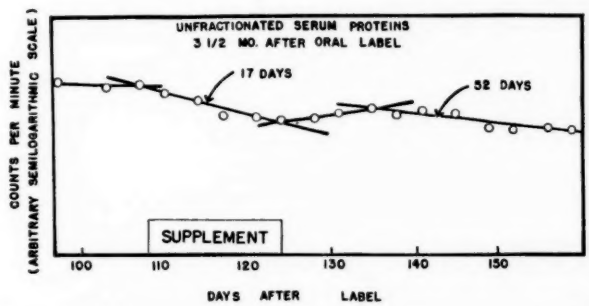

Fig. 5.—Patient T. C. Effect on die-away plot of oral unlabeled S amino acid supplementation started 110 days after oral dose of S^{35} amino acid in hypoproteinemic subject.

Because of low specific radioactivities, fractionation was impractical.

Upon discontinuation of the supplement, the die-away plot for both patients took a sharp upward swing, and then resumed the previous slow steady decline, so that a superimposed straight edge connects the first and last portions of the plot.

Fractionated serum proteins eighteen days after oral labeling with S^{35} methionine: In a second study in Patient J. M., the supplement was begun 18 days after oral labeling. The findings (Fig. 4, bottom) are quite different.

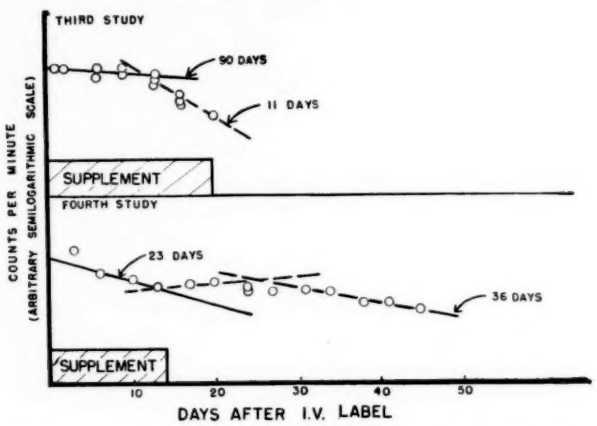

Fig. 6.—Patient J. M. Effect of immediate oral supplementation (5 Gm. cysteine, 5 Gm. methionine per day) on albumin die-away plots following intravenous S^{35} cysteine (same metabolically normal subject as in Fig. 4).

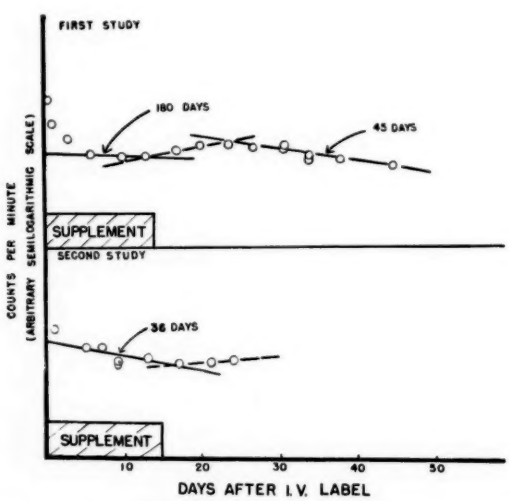

Fig. 7.—Patient R. S. Effect of immediate oral supplementation (5 Gm. cysteine, 5 Gm. methionine per day) on albumin die-away plots following intravenous S^{35} cysteine in a second metabolically normal subject.

The initial upward albumins slope and flat gamma globulins slope, prior to the administration of the supplement, suggests intensive isotope reutilization during this period. More important are these findings: (1) the albumins slope turns downward *before* the supplement is started; (2) the slope flattens rather than steepens during the supplement administration; and (3) neither the albumins nor the gamma globulins slope becomes as steep as the slope for unfractionated plasma proteins (Fig. 4, top) when the supplement is given 275 days after the oral label.

Following the analogy of Schoenheimer and his group's classic studies on N^{15},[9] these findings suggest that only after the oral label has become well distributed through the body's tissue protein stores (whose turnover rates are very slow) can recycling be repressed by oral unlabeled supplements. Such distribution takes months, not days. When, after but two weeks or so, such organs as the liver have specific activities about as high as those of simultaneously obtained plasma proteins,[2] oral supplements at the dosage used seem ineffective.

Albumins immediately after intravenous labeling with S^{35} cysteine: The above results suggested that failure to minimize recycling might still be due to several possibilities, namely, (1) the oral supplement was too small; (2) it was given too late after the initial oral label; and (3) that the intravenous route (by initial partial liver circumvention) might be preferable to the oral route for labeling.

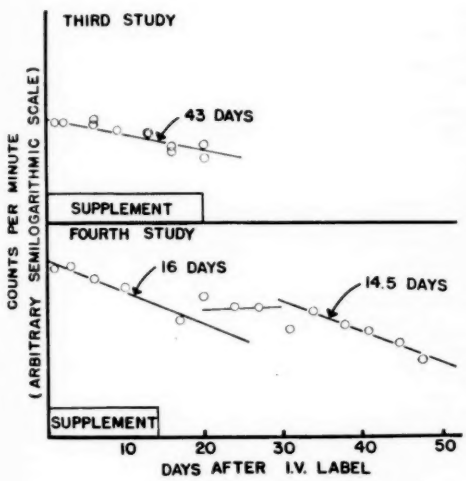

Fig. 8.

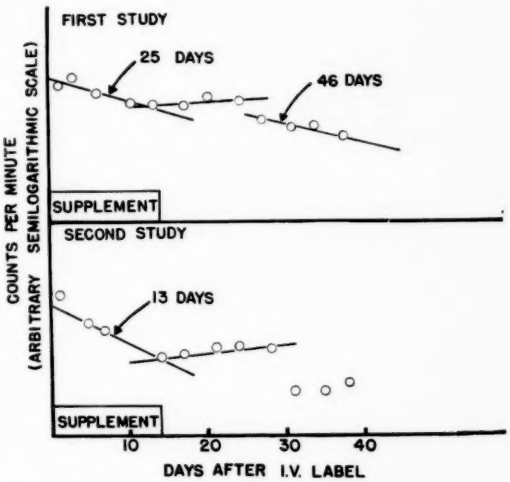

Fig. 9.

Fig. 8.—Patient J. M. Effect of immediate oral supplementation of gamma globulin die-away plots following intravenous S^{35} cysteine. (Compare to simultaneous study of albumins in the same subject, Fig. 6.

Fig. 9.—Patient R. S. Effect of immediate oral supplementation on gamma globulin die-away plots following intravenous S^{35} cysteine. (Compare to simultaneous study of albumins in the same subject, Fig. 7.

Figs. 6 and 7 present duplicate studies in the two normal patients for the albumin die-away plots when the supplement was given immediately following intravenous labeling with S^{35} cysteine. It will be noted that (1) there is no consistency in comparison of the apparent turnover times or the shapes of the die-away plots, either between the two patients or between duplicate studies in the same patient, although their clinical status during the study remained unchanged; (2) the shortest apparent turnover time, 23 days, in Patient J. M. (Fig. 6, bottom) was still six days longer than the figure obtained for albumins with the intravenous I^{131} label; (3) when sufficient residual radioactivity remained to permit extended observation, a rise in the slope followed by a gradual decrement occurred when the supplement was discontinued. The resultant roller-coaster appearance of the die-away plots is illustrated in Figs. 6

(bottom), 7 (top) and 8 (bottom); (4) the apparent turnover time characteristic of the terminal portion of the die-away plot in the fourth study of Patient J. M. (Fig. 6, bottom) was somewhat longer than during the second week after labeling. The reverse of this relationship is seen in the first study on R. S. (Fig. 7, top).

Gamma globulins immediately after intravenous labeling with S^{35} cysteine: Figs. 8 and 9 illustrate the gamma globulin die-away plots obtained simultaneously with the above albumin studies. The same remarks pertaining to the albumins are applicable here.

TABLE III. APPARENT TURNOVER TIMES OF ALBUMINS AND GLOBULINS IN A NORMAL PATIENT (J. M.) OBTAINED BY DIFFERENT LABELING PROCEDURES

METHODS OF LABELING	PERIOD STUDIED	APPARENT TURNOVER TIME (DAYS)	
		ALBUMINS	GAMMA GLOBULINS
S^{35} oral yeast, no supplement	7th-40th day	64	40
S^{35} oral cysteine, no supplement	7th-18th day	28	41
S^{35} oral cysteine, with supplement	18th-40th day	64	53
I^{131} intravenous fractions, Lugol's supplement	1st-20th day	17	12

DISCUSSION

The dilemma in the interpretation is well illustrated by Table III, in which apparent turnover times for the albumins and gamma globulins of a single subject, as measured by various labeling and supplement schedules, are summarized.

The discouraging implications for a valid method to determine turnover times of autosynthesized plasma proteins in clinical investigation clearly are not limited to the S^{35} level alone; the C^{14} and the N^{15} labels, to a greater or lesser degree, probably lead to the same methodological morass.

That the use of unlabeled amino acid supplements has failed, in the experiments described in this paper, to influence this unfavorable picture is perhaps not surprising from the recent animal work of Loftfield and Harris.[10] In their important paper, which summarizes and adds new evidence for the view that protein synthesis occurs directly from an intracellular free amino acid pool, they also bring evidence that in the rat liver, at least, this pool does not enjoy immediate equilibration with circulating free plasma amino acids, L-valine C^{14}, L-isoleucine C^{14}, and L-leucine C^{14} were used. Working with the responses of the ferritin synthesis system to iron stimulation, they failed to demonstrate any influence of massive intravenous injection of *unlabeled* amino acids on the specific radioactivity of the newly synthesized ferritin.

SUMMARY AND CONCLUSIONS

In this study, we have extended our previously reported comparisons, in the same subjects, of apparent turnover times of serum albumin and gamma globulin, autosynthesized following oral S^{35} ingestion, or intravenously injected following in vitro I^{131} or in vivo S^{35} labeling to two new situations: (1)

the intravenous injection of the labeling amino acid; and (2) the administration of large amounts of unlabeled S amino acids at various periods during the die-away plot in an effort to minimize isotope reutilization or "recycling."

The use of S^{35} cysteine, whether given orally or intravenously, whether or not accompanied by oral supplements of unlabeled S containing amino acids in dosage as high as 10 Gm. per day, has provided die-away plots for albumins and gamma globulins whose slopes do *not* have sufficient consistency or reproducibility to permit valid metabolic interpretation in terms of apparent turnover times for serum albumins or gamma globulins. Even with immediate unlabeled supplement, the results are only *slightly* more satisfactory than following the oral S^{35} methionine label. The discrepancies in apparent turnover times between autosynthesized and in vitro or in vivo label intravenously injected fractions have *not* been eliminated.

While an apparent effect of oral supplementation has been noted on the slope of the die-away plots some *months* after the oral S^{35} amino acid label, a method based on this tenuous finding would be hardly of value for clinical investigation. As a rule, one wants turnover data in the immediate future and not after months in which unpredictable clinical phenomena can occur.

In our opinion, these results make it very unlikely that oral isotope administration, except in the most crude empirical fashion, is going to prove a valid tool for the study of protein turnover in disease states.

We are grateful to Miss G. Robey for technical assistance and to Miss J. Peters of the Neurological Service at Cook County Hospital for metabolic nursing assistance.

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THE SIGNIFICANCE OF INTERACTIONS BETWEEN PROTEINS AND LIPIDS FOR SEROLOGIC CANCER DIAGNOSIS

MARTIN E. HANKE, PH.D., AND HERBERT KAHN, M.D.*
CHICAGO, ILL.

IN A previous study[1] the values of 2 protein fractions were measured on 578 sera obtained from the Chicago Lying-in Hospital. The serum proteins were precipitated onto strips of filter paper and extracted with 38.5 and 31 (w/v) per cent ammonium sulfate solutions. The first fraction, considered to be a more soluble albumin, was designated Fract. S; the proteins extracted with the 31 per cent solution approximate the total albumin. It was observed that at the same total albumin level the average Fract. S values were significantly lower in sera of patients with active malignancies (called cancer sera) than in sera of patients with nonmalignant diseases (called noncancer sera). See also Table VI of this paper. Some of the individual Fract. S values showed overlapping between the 2 groups of sera.

These experiments have now been extended. A study was made of factors which affect the magnitude of the Fract. S values, and which cause the differences between cancer and noncancer sera. Purified albumin solutions prepared from both groups of sera do not differ significantly in their Fract. S values. Addition to serum in vitro of globulin or certain steroids causes lowering of the Fract. S values, while addition of fatty acids with 6 to 12 carbon atoms has an elevating effect (Table I). Most of the lipids extracted from sera have no significant effect; however, a lipid fraction extracted from cancer sera, which is soluble in ether, acetone, and cold methanol, regularly causes lowering of the Fract. S values.

Previously we had concluded that cancer sera have a characteristic deficiency of the more soluble albumin fraction.[2-4] The results of the experiments which are summarized in Table I indicate that the differences between cancer and noncancer sera are based not on variations in the quantity or nature of the serum proteins per se, but on differences in the interactions between proteins and certain nonprotein substances, especially lipids.

In this study, centrifugation was used for separating dissolved from solid protein fractions instead of paper extraction employed in the previous study.[1]

From the Department of Biochemistry, University of Chicago, Chicago, Ill.

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Some of the more recent studies were carried out by one of us (K.) in the Department of Clinical Science, University of Illinois College of Medicine, Chicago.

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*Present address: Department of Clinical Science, University of Illinois College of Medicine, Chicago 12, Ill.

The serum proteins were precipitated at a concentration of 42.6 per cent ammonium sulfate and, after centrifugation, were extracted successively with three more dilute ammonium sulfate solutions, so as to determine two albumin* fractions and globulin.

TABLE I. ALTERING EFFECTS OF SEVERAL SUBSTANCES ON FRACT. S VALUES*

ADDED SUBSTANCE†	NUMBER OF EXPERIMENTS	CONCENTRATION PER ML. SERUM	AVERAGE EFFECT PER CENT‡
Human serum globulins	8	15	−30
Cholic acid	2	2	−33
Glycodesoxycholic acid	8	2	−42
Dehydrocholic acid	4	1	−49
Progesterone	2	1	−25
Testosterone	2	1	−36
Dehydroepiandrosterone	6	1	−55
Diethylstilbestrol	2	0.3	−24
Sodium caprylate	10	1	+30
Sodium laurate	10	2	+104
Lipids§ from cancer sera	25	2	−18
from noncancer sera	25	2	−7

No altering effects were observed after addition of most inorganic salts (which do not precipitate proteins), sugars, amino acids, nucleic acids, B vitamins, calciferol, cholesterol, lithocholic acid, cortisone, estradiol, and methylcholanthrene.

*Fract. S is the more readily extractable albumin fraction by the paper extraction method.[1]

†Serum was added to a weighed amount of the substance and stirred with a glass rod until complete solution or homogenous distribution was achieved.

‡The altering effects were calculated as

$$100\left(\frac{\text{Fract. S of modified serum}}{\text{Fract. S of unmodified serum}} - 1\right)$$

The magnitude of the effects varied in different sera. The averages are given.

§These lipids were soluble in acetone and cold methanol. For details see text.

METHODS AND MATERIALS

Most of the sera used in this series were obtained from the Department of Surgery of the University of Chicago Clinics. All diagnoses of malignancies were confirmed histologically. The blood was drawn in the postabsorptive stage. The sera could be stored in the refrigerator for one week. Hemolysis and freezing were avoided.

As described previously,[1] the ammonium sulfate solutions were buffered with 0.07 M ammonium phosphate. The percentage number indicates grams of $(NH_4)_2SO_4$ in 100 ml. of solution.

Procedure.—All estimations were carried out in duplicate at 5° C. The mixtures of sera (or protein solutions) and ammonium sulfate solutions were stirred with a glass rod until a homogeneous distribution of the precipitates was achieved. While standing, the tubes were stoppered tightly with rubber stoppers. Before centrifugation the sediment was redistributed by whirling. We used a Servall centrifuge at 12,000 r.p.m. for 20 minutes. It is advisable to include one normal serum in each series in order to certify the consistency of experimental conditions.

A. Measure 0.5 ml. serum (plasma or protein solution) into a 15 ml. plastic tube, add 4 ml. of 48 per cent $(NH_4)_2SO_4$ solution pH 7.3, mix, let stand for one hour, and centrifuge but do not decant. Add 1 ml. of a 18.1 per cent $(NH_4)_2SO_4$ solution pH 7.3, which brings the $(NH_4)_2SO_4$ concentration of the mixture to 38.2 per cent. Stir and let stand for 18 to 20 hours. Centrifuge and decant the supernatant into a glass tube. Mix 4 ml. of the clear

*Originally we separated the albumin into 4 different fractions. Evaluation of the clinical data indicated that the first albumin fraction has primary significance for cancer diagnosis. Control experiments on the same sera showed that the sum of the 4 albumin fractions estimated with the more extensive method agreed with the sum of the 2 albumin fractions estimated with the described method within the limits of experimental error.

supernatant* with 11 ml. of a 31 per cent $(NH_4)_2SO_4$ solution at pH 6.8 in a 20 ml. test tube. Heat the mixture for 3 minutes in a boiling water bath, and, after cooling, read the turbidity in a photoelectric colorimeter with a 420 mμ filter.

B. Add to precipitate A, 10 ml. of a 31 per cent $(NH_4)_2SO_4$ solution pH 6.8 and stir. Measure 1 ml. of this mixture into a 50 ml. plastic tube, mix with 14 ml. of 31 per cent $(NH_4)_2SO_4$ solution pH 6.8, and stir. Let stand for at least 2 hours. Centrifuge, decant the supernatant into a 20 ml. test tube, heat, and read the turbidity as in A.

C. Add to the precipitate B in the 50 ml. plastic tube 15 ml. of 12 per cent $(NH_4)_2SO_4$ solution pH 6.8, stir, and let stand for at least one hour. Centrifuge, decant the supernatant into a 20 ml. test tube, heat, and read the turbidity as above.

The photoelectric colorimeter was calibrated with purified albumin solutions for extracts A and B and with globulin solutions for extracts C. The protein concentrations were calculated as grams per 100 ml. of serum or original protein solution. The calculated values from extract A were designated as Fract. A. The sum of the values of extracts A and B approximates the total albumin and was designated as TA. The values from extracts C represent the globulins, and were designated as GL. Any fibrinogen which may have been present remained in the precipitate C and was discarded.

On the average, 100 ml. of normal serum contained 0.49 Gm. of Fract. A or 11 per cent of TA. The corresponding Fract. S values were 1.12 Gm. or 25 per cent of TA.[1]

The effect of serum-lipid fractions on Fract. A values was measured by the following procedure. The sera were extracted with a 3 to 1 ethanol-ether mixture. After centrifugation the supernatant was evaporated in vacuo and the residue extracted with petroleum ether. The residue from the evaporation of the petroleum ether extract was extracted at −3° C. with acetone. After centrifugation the supernatant was evaporated in vacuo. An aliquot of the residue, corresponding to 2 ml. of the extracted serum, was mixed with 2 ml. of a test serum by stirring with a glass rod. After standing overnight in the cold, the mixture was centrifuged vigorously so that an almost clear middle layer was obtained, which was used for the analysis of Fract. A. Many of the supernatants A showed slight turbidities; they were decanted and clarified by an additional centrifugation before they were measured for the protein estimation.*

RESULTS

The ratios Fract. A/TA increase in sera as TA is decreased by dilution, but the Fract. A values show little or no change (Table II) at a dilution of the serum of 1:2. Increase of GL concentration decreases the Fract. A values. This decrease seems to be proportional to the increase of the ratios GL/TA, and the products Fract. A $\frac{TA + GL}{TA}$ vary insignificantly for different GL concentrations (Table II). Some of the substances which altered Fract. S (Table I) were tested with the centrifugation method. Comparison of Tables I and II shows that substances tested by both methods alter Fract. S and Fract. A in the same direction.

The data in Table III show a certain parallelism between the value of the products Fract. A $\frac{TA + GL}{TA}$ of sera and the magnitude of the lowering effect of the lipid fraction which was extracted from those sera. This indicates that at least a part of the variations in the values of these products is caused by

*More recent studies have shown that the accuracy of the method is increased and its applicability extended to very turbid sera (which had to be excluded from this study) when all sera are clarified routinely by centrifugation at high speed before the analysis and when the decanted supernatants A are centrifuged again before the protein estimation.

interactions of proteins and lipids. Comparison of Fract. A values of purified albumin solutions (from which the lipids have been removed) prepared from cancer and from noncancer sera revealed no significant differences.

TABLE II. EFFECTS OF IN VITRO MODIFICATIONS OF SERA OR HUMAN ALBUMIN SOLUTIONS ON THEIR FRACT. A. VALUES*

	FRACT. A* (GM. IN 100 ML.)	TA* (GM. IN 100 ML.)	GL* (GM. IN 100 ML.)	CHANGE IN FRACT. A PER CENT	FRACT. A $\frac{TA + GL}{TA}$ (GM. IN 100 ML.)
I. Dilution:					
Serum	0.50	4.68	2.82		0.80
Serum + equal volume of water	0.50	2.33	1.40		0.80
II. Addition of pseudoglobulin†					
A. Serum albumin solution	0.39	3.21	0.00		0.39
B. Albumin-pseudoglobulin mixture	0.25	3.23	1.63	−36	0.38
C. Equal parts of A and B	0.32	3.32	0.74	−18	0.39
D. One part of B and 3 parts of A	0.36	3.32	0.40	−8	0.39
III. Addition of nonprotein substances:					
Serum alone	0.47	4.16	4.50		0.98
Serum plus 1.3 mg. of sodium caprylate per ml.	0.85‡	4.31	4.16	+81	1.67
Serum plus 0.7 mg. of dehydrocholic acid per ml.	0.10	4.09	4.50	−78	0.21
Serum plus 0.2 mg. of dehydroepiandrosterone per ml.	0.33	4.19	4.34	−30	0.66
IV. Addition of serum lipids:					
Serum alone	0.37	4.20	3.10		0.64
+ lipids§ from a noncancer serum	0.35	4.20	3.10	−5	0.61
+ lipids§ from a cancer serum	0.31	4.20	3.10	−16	0.54

*Fract. A is the more readily extractable albumin by the contrifugation method described in the text. TA means total albumin; GL, globulin.

†Albumin and pseudoglobulin were prepared from pooled human sera by fractionation with ammonium sulfate and dialysis.

‡In this case the supernatant was turbid.

§These lipid fractions were soluble in petroleum ether and cold acetone. For details see text.

TABLE III. COMPARISON OF CORRECTED FRACT. A* VALUES OF SEVERAL GROUPS OF SERA AND FRACT. A-LOWERING EFFECTS OF THEIR LIPIDS†

SERA FROM PATIENTS	FRACT. A $\frac{TA + GL^*}{TA}$ (GM. PER 100 ML.) RANGE	FRACT. A $\frac{TA + GL^*}{TA}$ (GM. PER 100 ML.) AVERAGE	CHANGE OF FRACT. A VALUES AFTER MIXING A NORMAL SERUM WITH LIPIDS† FROM THESE SERA (PER CENT) RANGE	CHANGE OF FRACT. A VALUES AFTER MIXING A NORMAL SERUM WITH LIPIDS† FROM THESE SERA (PER CENT) AVERAGE
3 without malignancies	0.73 to 0.61	0.66	+3 to −10	−4
4 with treated malignancies	0.64 to 0.54	0.58	−6 to −10	−7
4 with active malignancies	0.48 to 0.30	0.41	−16 to −20	−18

*For the meaning of Fract. A, see text. TA means total albumin; GL, globulin.

†For preparation of these lipids, see text.

The data on cancer and noncancer sera are arranged separately in order of their Fract. A values in Tables IV and V. To facilitate the evaluation of the relations of the 3 protein fractions and to make a comparison possible with the previous clinical study,[1] Table VI was prepared by segregating the data of Tables IV and V into 3 TA ranges (above 90 per cent, 70 to 89 per cent, and 60 to 69 per cent of the average normal TA of 4.5 Gm. per 100 ml. serum) and computing the average Fract. A values of each range. The Fract. A values decrease in the lower TA ranges, but the average ratios Fract. A/TA remain

TABLE V. FRACT. A, TOTAL ALBUMIN (TA) AND GLOBULIN (GL) IN SERA FROM 24 NONMALIGNANCIES

NAME	DIAGNOSIS	FRACT. A	TA	GL	FRACT. A $\frac{TA+GL}{TA}$
		(GM. IN 100 ML. SERUM)			
Ca	Latent syphilis	0.53	4.33	3.40	0.95
Ki	Neurasthenia	0.50	4.68	2.82	0.80
Ha	Normal	0.45	4.17	3.72	0.85
Ne	Thyroid disease	0.44	4.48	3.76	0.81
St	Uveitis	0.44	3.92	3.39	0.82
Me	Arthritis	0.43	4.49	3.52	0.77
Dod	Esophagitis	0.43	4.06	3.08	0.77
Ki	Adenoma of breast	0.41	3.16	3.80	0.90
Kr	Biliary cirrhosis	0.41	2.78	4.12	1.02
Do	Banti's syndrome	0.40	4.28	4.70	0.83
La	Uveitis	0.39	4.13	4.09	0.78
El	Thyroid disease	0.39	3.99	3.08	0.69
Ne	Thyroid disease	0.39	3.91	3.76	0.77
Do	Hypothyroid	0.38	4.13	2.59	0.62
Mo	Hypothyroid	0.36	3.59	3.70	0.73
Wa	Hypertrophy of prostate	0.35	4.26	4.13	0.69
Ba	Diabetes mellitus	0.34	3.81	3.56	0.66
Hu	Iritis	0.34	3.73	3.60	0.65
Ste	Cushing's syndrome	0.33	3.68	3.42	0.64
Car	Cirrhosis of liver	0.32	2.92	4.28	0.79
Schu	Stenosis of rectum; diverticulitis	0.25	3.12	4.40	0.61
She	Fibrosis of lungs	0.18	3.13	8.41	0.66
Fo	Abscess of gall bladder; diabetes, nephritis	0.10	1.88	4.36	0.33
	Average	0.37	3.78	3.88	0.75

TABLE V. FRACT. A, TOTAL ALBUMIN (TA), AND GLOBULIN (GL) IN SERA FROM 19 ACTIVE CARCINOMAS

NAME	TYPE	PRIMARY IN	FRACT. A	TA	GL	FRACT. A $\frac{TA+GL}{TA}$
			(GM. IN 100 ML. SERUM)			
Phi	Adeno.	Rectum	0.33	4.26	2.82	0.55
Du	Adeno.	Breast	0.32	4.39	2.56	0.51
Ga	Squamous cell	Soft palate	0.31	4.73	2.34	0.47
Tr	Adeno.	Breast	0.26	3.67	3.46	0.51
Bi	Adeno.	Breast	0.25	3.77	4.82	0.57
Eh	Adeno.	Breast	0.24	3.71	2.50	0.40
Le	Scirrhous	Stomach	0.24	2.67	4.00	0.60
An	Adeno.	Colon	0.22	2.94	1.76	0.35
B. J.	Adeno.	Sigmoid	0.21	3.82	4.46	0.45
Ro	Adeno.	Adrenal	0.21	3.15	2.82	0.40
Ma	Adeno.	Esophagus	0.21	2.64	3.84	0.52
Ab	Adeno.	Breast	0.19	2.76	4.00	0.47
La	Adeno.	Breast	0.18	2.75	2.00	0.31
Le	Adeno.	Breast	0.18	2.75	2.32	0.33
Je	Adeno.	Sigmoid	0.14	2.66	3.30	0.32
Fr	Adeno.	Breast	0.13	2.99	6.29	0.40
Pe	Adeno.	Lung	0.10	2.57	3.88	0.25
B. H.	Adeno.	Breast	0.10	2.52	3.20	0.23
Ho	Adeno.	Colon	0.10	1.89	2.52	0.23
	Average		0.22	3.15	3.34	0.45
Pi	Multiple myeloma		0.08	1.00	5.20	0.50
Ma	Adeno.	Breast	0.34	4.18	3.82	0.65
	Clinically no recurrence, one year after surgery					

fairly constant in each group of sera. The products, Fract. A $\frac{TA + GL}{TA}$, show only small variations in the 3 TA ranges for each of the noncancer and cancer groups. After the effects of the globulin concentrations were corrected by calculating the products Fract. A $\frac{TA + GL}{TA}$ a clear separation of the noncancer and of the cancer sera becomes possible. As dividing point was chosen half of the sum of the grand averages of the products of the noncancer and of the cancer sera (Table VI): $\frac{0.77 + 0.44}{2} = 0.605$. All 20 sera from active malignancies have lower products (Table V), and 23 of the 24 noncancer sera have higher products (Table IV). Allowing for ± 5 per cent experimental error, 4 sera (3 noncancer and 1 cancer) fall in the intermediate zone of 0.575 to 0.635.

TABLE VI. COMPARISON OF THE AVERAGE VALUES OF FRACT. A AND OF THE PRODUCTS FRACT. A $\frac{TA + GL}{TA}$ IN 3 TA RANGES FOR NONCANCER AND CANCER SERA

TA RANGE	FRACT. A*	TA*	GL*	RATIO OF FRACT. A TO TA	FRACT. A $\frac{TA + GL}{TA}$
	(GM. IN 100 ML. SERUM)				(GM. IN 100 ML. SERUM)
			Nonmalignancies		
Above 4.05	0.43	4.30	3.58	0.10	0.79
3.15 to 4.04	0.37	3.73	3.50	0.10	0.72
2.70 to 3.14	0.29	2.98	5.30	0.10	0.81
Average	0.36	3.67	4.13	0.10	0.77
			Malignancies		
Above 4.05	0.32	4.46	2.57	0.07	0.50
3.15 to 0.04	0.23	3.61	3.26	0.06	0.44
2.70 to 3.14	0.19	2.85	3.27	0.07	0.41
Average	0.25	3.64	3.03	0.07	0.44

*For the meaning of Fract. A, see text. TA means total albumin; GL, globulin.

TABLE VII. COMPARISON BETWEEN THE RESULTS OBTAINED ON SERA FROM NONMALIGNANCIES AND FROM MALIGNANCIES BY THE CENTRIFUGATION METHOD (CM) AND BY THE PAPER EXTRACTION METHOD (PM)

TA RANGE (GM. IN 100 ML. SERUM)	NONMALIGNANCIES			MALIGNANCIES		
	TA (GM. IN 100 ML. SERUM)	S (% OF NORMAL)		TA (GM. IN 100 ML. SERUM)	S (% OF NORMAL)	
		CM	PM		CM	PM
Above 4.05	4.30	88	88	4.46	65	69
3.15 to 0.04	3.73	75	78	3.61	47	57
2.70 to 3.14	2.98	59	58	2.85	39	40
Grand average	3.67	74	75	3.64	50	55

The averages for the Fract. A values in the 3 total albumin (TA) ranges in Table IV were transformed into S values, i.e., per cent of normal values by the formula S = 100 × Fract. A/0.49. The S values for the same total albumin values were read from Fig. 1 of the previous paper.[1]

Comparison Between Evaluations of Results Obtained With the Centrifugation Method and With the Paper Extraction Method.—Since our experiments had to be interrupted, we can report data only from 45 sera, analyzed with the centrifugation method. Our conclusions can be supported by the results on

578 sera analyzed previously with the paper extraction method.[1] Those data had been reported as S values, i.e., as per cent of the average Fract. S values of normal sera. To make comparable the results obtained with both methods, the Fract. A values from Table VI were calculated as S values, that is, per cent of normal by the formula 100 × Fract. A/0.49. Table VII shows that the average S values of the noncancer sera are very similar by both methods at each level of total albumin; while in the cancer sera the values by the centrifugation method are about 10 per cent lower than by the paper extraction method.

The Nature of Fract. A, a More Rapidly Extractable Albumin.—The magnitude of the Fract. A value of any serum gradually decreases as the time of standing of the mixture of serum with 38.2 per cent $(NH_4)_2SO_4$ is increased. After 1 hour the value is about 15 per cent greater, and after 48 hours about 10 per cent less, than the value at 19 hours. Even after 9 days, when the value has decreased about half, it continues to decrease. Also vigorous stirring or any elevation of temperature up to 35° C. decreases Fract. A values. It should be emphasized that such variations in time, agitation, or temperature have no significant effect on the total albumin value as measured by 31 per cent $(NH_4)_2SO_4$ or on the globulin values measured at 12 per cent $(NH_4)_2SO_4$, and this supports the idea that these decreases in Fract. A are not due to denaturation of protein.

The decreasing effect on the magnitude of Fract. A of increased duration of extraction and stirring demonstrates that Fract. A is not, as we formerly thought,[1, 5] a more soluble albumin, but rather a more rapidly extractable albumin fraction. The manner in which the presence of lipids or of globulin alters the *rate* of albumin solution is not clear, but the phenomenon undoubtedly involves interaction of albumin with lipids or albumin with globulin.

DISCUSSION

In the previous study a separation of the Fract. S values of cancer and noncancer sera was made in terms of a dividing line constructed from the average S values of each group at different TA levels.[1] In the present study the consideration of another factor, the globulin concentration, leads to a simpler and more accurate evaluation. One value, the product Fract. A $\frac{TA + GL}{TA}$, is used as dividing point for all sera. The data in Table II give an experimental basis for the use of this product. It represents the Fract. A values with correction for the effects of variations in the ratios GL/TA.

The centrifugation method described in this paper for estimation of Fract. A has the following advantages when compared with the paper extraction method which was used for measuring Fract. S. The use of paper is eliminated; so the effects of interaction between paper and proteins are avoided. The ratio of serum to extracting solution is increased and a smaller percentage of the albumin is extracted; these allow a more accurate measurement of the effects of some Fract. A altering substances. The effects of temperature variations on the amount of extracted protein are minimized. The data show less overlapping in the corrected values for the individual noncancer and cancer sera.

Disadvantages of the centrifugation method are the requirement of some special equipment and more time. In some sera the supernatants A were turbid before heating, and the method could not be applied.* We observed this phenomenon in 2 noncancer sera which were excluded from Table IV.

The effects of the addition of various lipids to serum in vitro (Tables I, II, and III) are best interpreted by assuming that the Fract. S and Fract. A values are influenced markedly by interactions between serum proteins and lipids. One may conclude that in sera of the same albumin and globulin concentration, different Fract. A values under various physiologic and pathologic conditions reflect variations in the concentration of Fract. A-elevating or Fract. A-lowering lipids. The lower-than-normal Fract. A values usually observed in sera of diseased persons indicates either an increase in Fract. A-lowering or a decrease in Fract. A-elevating substances. Further investigations are necessary to determine whether the still-lower Fract. A values in malignancies are caused by an increase in the concentration of the same kind of Fract. A-lowering substances commonly present in sera, or to the presence of specific lipids characteristic of malignant growth.

We wish to emphasize that the described method is nonspecific and not a "cancer" test. It is recommended not as a substitute but rather as a supplement to cytological methods. Cytology reveals the presence of abnormal cells. Serology measures chemical changes caused by their presence and is complicated by interfering factors related to other pathologic conditions. We feel that analyses of a greater number of unselected sera from the same kinds of patients would not change significantly the statistical results. To determine the clinical value of the proposed method for cancer diagnosis it seems more important to re-examine with this method selected sera of patients with certain conditions in which "false positive" results had been obtained with previous methods, e.g., certain chronic or acute infections, liver and kidney diseases, and pregnancy. The effects of food intake and of certain drugs (e.g., sulfa drugs) have to be established. The duration and magnitude of aftereffects of radiation and surgery on Fract. A values must be determined before it will be possible to evaluate the significance of Fract. A data on sera of treated cancer patients. When information on these variables is available, the proposed method may be a useful aid in the diagnosis and prognosis of cancer.

This method should be useful, along with other methods, in the characterization and isolation of Fract. A-lowering lipids or lipid-protein complexes which are present during malignant growth. If such lipids are found to be characteristic of certain or all kinds of malignancy, the significance would be great not only for serodiagnosis of cancer, but also for the metabolic nature of malignant growth.

SUMMARY

1. A method is described in which the serum proteins are precipitated and then extracted successively with 38.2, 31, and 12 per cent ammonium sulfate solutions. The extracts are separated from the precipitates by centrifugation at high speed, and their protein concentrations are measured turbidimetrically.

*See footnote on page 360.

They are designated as Fract. A, B, and C, respectively. The sum of Fract. A and Fract. B approximates the total albumin (TA) and Fract. C, the globulin (GL).

2. With this method 45 sera (20 from malignancies) were analyzed. It was found that the products Fract. A $\frac{TA + GL}{TA}$ for all sera of patients with malignant disease were lower than a certain reference value, while those of patients without malignant disease, with one exception, were higher.

3. These results are compared with the evaluation of analyses on 578 sera in which the serum proteins had been precipitated onto strips of filter paper, and extracted with 38.5 and 31 per cent ammonium sulfate solutions. The advantages of the centrifugation method are discussed.

4. In vitro studies indicate that the characteristic differences between sera of patients with and without malignancies are due not to variations in the quantity or nature of the serum proteins, but to their interactions with certain nonprotein substances. Mixing sera with certain fatty acids causes elevation of their Fract. A values. Lower Fract. A values are observed after mixing sera with most bile acids, with certain steroids and other substances related to sex hormones, or with certain acetone-soluble lipids extracted from cancer sera.

5. These observations suggest that during malignant growth certain characteristic lipids or lipid-protein combinations occur in the serum.

6. The clinical value and the limitations of the described method for serologic cancer diagnosis and prognosis are discussed.

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CHROMOGENS IN THE URINE OF NORMAL INDIVIDUALS AND OF EPILEPTIC PATIENTS WHICH REACT WITH EHRLICH'S ALDEHYDE REAGENT

MEYER MARKOVITZ, M.D.*
CHICAGO, ILL.

THE test for porphobilinogen described by Watson and Schwartz[1] in 1941 has gained widespread acceptance as a simple laboratory aid in the diagnosis of acute intermittent porphyria. Hammond and Welcher[2] concluded that the test was quite specific.

For several years I was interested in following a number of patients with acute intermittent porphyria.[3] As a result of this interest, 2 patients were referred from the Neurology-Epilepsy Clinic. These patients had been treated for epilepsy for over 5 years, and had recently developed acute, intermittent abdominal pain. The diagnosis of acute intermittent porphyria was entertained, especially when a test of their urine for porphobilinogen revealed a purplish-pink color which was insoluble in chloroform. However, no uroporphyrin and no increase in coproporphyrin could be detected in several 24-hour specimens of the patients' urine.

Two other possibilities came to mind. First, that the barbiturates used in the treatment of epilepsy might have caused some aberration in pyrrole metabolism. This would fit nicely with the clinical observation of Waldenstrom[4] that barbiturates may cause exacerbations of acute intermittent porphyria, and the experimental evidence described by Goldberg[5] on the effect of certain barbiturates on the porphyrin metabolism of rabbits. The second possibility derived from the observation that a considerable number of patients with acute intermittent porphyria have epileptiform seizures. If the urine of patients with epilepsy contained porphobilinogen, then perhaps there was also a defect in pyrrole metabolism in epilepsy. (In this connection, Goldberg and his associates[6] have recently discussed the lack of physiologic activity of purified porphobilinogen.)

This is a report concerning the frequent finding of a positive test for porphobilinogen in the urine of epileptic patients, and attempts to characterize the chromogen responsible.

METHODS

The test for porphobilinogen was performed as described below. Five milliliters of Ehrlich's aldehyde reagent (as modified by Watson) was added to 5 ml. of fresh urine. Ten milliliters of a saturated solution of sodium acetate was then added. Finally, 5 ml. of

From the Neurophysiology Laboratories, Department of Psychiatry, University of Illinois College of Medicine, Chicago, Ill.
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*Present address: 725 West Lewis, Phoenix, Ariz.

chloroform was added. The mixture was shaken well after each addition. The test was considered negative if no pink color remained in the aqueous phase. Only a definite purplish-pink color was considered positive. Reagents from several different sources were used with identical results.

Later in the study, the Vahlquist type of Ehrlich's aldehyde reagent was used. This necessitated filtering after the addition of the saturated solution of sodium acetate.

The method used to concentrate the Ehrlich aldehyde reacting substances in the urine was a modification of the methods of Westall,[7] and of Cookson and Rimington[8] for the crystallization of porphobilinogen.

Urine specimens were collected daily for 3 to 6 days from 5 normal individuals (fellow laboratory workers, all men, and under age 35) and 5 epileptic patients.* Each day's specimen was acidified with acetic acid, and then mercuric acetate (15 per cent w/v) was added until precipitation was complete. The precipitate was collected and washed with mercuric acetate (1 per cent w/v). The daily specimens were combined, resuspended in distilled water and decomposed by H_2S. After filtration and aeration, excess lead acetate (10 per cent w/v) was added. The deeply pigmented precipitate was removed and silver nitrate (20 per cent w/v) was added to the filtrate until precipitation was complete. The silver nitrate precipitate was removed and mercuric acetate (15 per cent w/v) was again added to the filtrate. This mercury precipitate, almost white, was collected, resuspended in a small amount of distilled water, and decomposed with H_2S. After filtration and aeration, the filtrate was passed through an alumina column, approximately 3 cm. in diameter, and 20 cm. in length, prepared by washing with 3 per cent acetic acid followed by 1,500 ml. of water. After all the filtrate had been put on the column, water was then passed through until no material giving a color with Ehrlich's aldehyde reagent appeared in the eluate. Ammonia, 0.1 N, was then run through the column, at a rate not exceeding 100 ml. per hour. The Ehrlich aldehyde reacting material in the eluate was collected on a fraction collector. The fractions were then lyophyllized, taken up in 5 ml. of 0.1 N ammonia, and then frozen.

Paper chromatograms were made by the ascending method and were run 4½ to 6 hours at room temperature. The solvent was a mixture of butanol, acetic acid, and water (using the top layer only) as described by Partridge.[9] The paper chromatograms were dried at room temperature and then sprayed with Vahlquist type Ehrlich's aldehyde reagent.

Urine specimens from two patients with porphyria hepatica, acute intermittent type, were prepared in the same manner and used for comparison of porphobilinogen with the chromogens present in the samples from epileptic patients.

RESULTS

Table I gives the results of the Watson-Schwartz tests in 91 patients from the neurology clinic.

Fourteen of these 91 epileptic patients (those with the most strongly "positive" tests for porphobilinogen) were then examined as to their liver function by means of Bromsulphalein excretion, thymol turbidity, cephalin flocculation, and serum bilirubin. All values for these tests were within normal limits except for a mild reduction in Bromsulphalein excretion in one patient. Twelve of the fourteen still had "positive" tests for porphobilinogen.

In the patients tested, there was no correlation of "positive" tests with the type of epilepsy, drug used, or age and sex of the patient. The only possible exception was a group of 5 patients with petit mal epilepsy alone, none of whom had a "positive" test. The drugs used in the treatment of these patients included phenobarbital, Dilantin, Tridione, Mesantoin, Mebaral, Milontin, Diamox, Phenurone, Mysoline, Eliptan, and Hibicon.

*Dr. Leo Kaminetz, Clinical Director, Dixon State School, Dixon, Ill.

Spectrophotometric studies of the purplish-pink color obtained when the urine was tested for porphobilinogen showed an abrupt increase in light transmission at 580 mμ, and a long, relatively flat plateau from 500 to 580 mμ, quite distinct from the characteristics of porphobilinogen aldehyde (552 and 522 mμ).

TABLE I. PATIENTS WITH EPILEPSY*

CLASSIFICATION	NUMBER OF PATIENTS	NUMBER OF TESTS "POSITIVE"
Total Patients	91	47
Male	54	33
Female	42	15
Under age 14	18	7
Over age 50	4	2
Petit mal	5	0
Petit mal + grand mal	26	11
Grand mal + psychomotor	39	25
Psychomotor	10	5
All other epilepsy	11	6

*Diagnoses are the result of a correlation of clinical and electroencephalographic findings.

The urine of the 5 epileptic patients at Dixon State Hospital and of the 5 fellow laboratory people was then treated by the method described above. In this attempt to concentrate and then characterize the chromogen responsible for the "positive" tests for porphobilinogen in the epileptic patients, three major fractions were obtained. The first, the water fraction, contained chromogen which would not adhere to the alumina column and was collected by washing the alumina column with water. The second fraction appeared at a pH of 4 to 6 when the column was washed with dilute ammonia. The third fraction was easily seen traveling down the alumina column as a brownish ring and appeared in the eluate at a pH of 7.

The water fraction gave a pinkish-purple color upon the addition of Ehrlich's aldehyde reagent. The addition of saturated sodium acetate solution made the color more purple. This purple color was insoluble and light absorption studies still showed a plateau from 540 to 570 mμ. Upon standing for several days at room temperature a grayish color developed.

The pH 4 to 6 fraction was found in 4 out of 5 normals but not in the epileptic patients. It produced an orange-brown color with Ehrlich's aldehyde reagent and became yellowish upon the addition of saturated sodium acetate solution. Spectrophotometric study of this pigment showed a plateau from 470 to 500 mμ.

The pH 7 fraction gave a purplish color upon addition of Ehrlich's aldehyde reagent, and addition of a saturated solution of sodium acetate produced a faint light blue color.

Ascending paper chromatograms were then made, using all of the fractions. As a control, the pH 7 fraction from a patient with acute intermittent porphyria was used. This fraction contained porphobilinogen and the aldehyde color gave a peak at 552 mμ. The purplish-pink spot of porphobilinogen aldehyde appeared immediately upon spraying with aldehyde reagent. When very small spots were made, this material had an R.F. of 0.51. The water fraction, in both normal and epileptic patients, yielded a purple spot with an R.F. of 0.50. This

purple spot appeared slowly after the paper was sprayed with the aldehyde reagent, and did not reach maximum intensity until about 15 minutes. A few hours later the spot became yellow and 24 hours later it was a blue-green.

The pH 4 to 6 fraction, found in 4 out of 5 normal urines but not in the urine of epileptic patients, yielded two spots, both orange-brown. The larger spot had an R.F. of 0.46 and appeared within one minute after spraying with the aldehyde reagent. Under ultraviolet light, this spot fluoresced a bright pinkish-red reminiscent of uroporphyrin. The smaller spot appeared slowly and had an R.F. of 0.34 and did not fluoresce.

The pH 7 fraction contained three spots in 3 out of 5 normal urines, but only two spots in all the urines of epileptic patients, as well as in two of the normal urines. All of these spots appeared slowly after spraying with the aldehyde reagent and reached maximum intensity over an hour later. Two of the spots were purplish and faint and had R.F. values of 0.95 and 0.78. The third spot, present in 3 out of 5 normals only, was most prominent. It was deep blue and had an R.F. of 0.86.

In two patients with acute intermittent porphyria, the same fractions present in the urine of normal patients were found, in addition to the porphobilinogen in the pH 7 fraction. In two additional patients with epilepsy, who were having frequent seizures when their urine was collected, very little material giving any color with Ehrlich's aldehyde reagent could be found. In one of these, no such pigment could be demonstrated at all, and in the other, only a small water fraction, but no pH 4-6 or pH 7 fractions could be found.

Under ultraviolet light a great many small spots and bands appeared in all of the fractions. Before spraying with aldehyde reagent many of these had a light bluish fluorescence. After spraying with aldehyde reagent only the porphobilinogen spot from the urine of patients with acute intermittent porphyria and the spot in the pH 4-6 fraction with an R.F. of 0.46 gave a pink fluorescence which looked like uroporphyrin.

DISCUSSION

The Watson-Schwartz test of the urine of epileptic patients was suggestive of a positive test in roughly 50 per cent of patients tested. Results of concentration of urine and chromatography of the concentrated fractions failed to reveal any porphobilinogen of the type found in acute intermittent porphyria. However, several chromogens were present in the concentrated urine of both normal and epileptic patients which could be confused with porphobilinogen upon the addition of Ehrlich's aldehyde reagent. Possibly, a quantitative difference exists in the chromogens present in the urine of normal versus epileptic patients which would account for the mildly positive-appearing tests in the unconcentrated urine of epileptic patients. It also seems likely that the amount of chromogens excreted in the urine of epileptic patients varies widely from time to time. The only qualitative differences were the absence of the pH 4-6

fraction from the urine of epileptic patients and also the absence of the R.F. 0.86 portion of the pH 7 fraction of the urine of these same patients. These fractions were found in the urine of normal patients.

The chromogens responsible for the colors when aldehyde reagent was added have not been identified. They are distinctly different from porphobilinogen, and may be indoles or tryptophane.[10] These chromogens may be a reflection of a metabolic defect causing disturbances in central nervous system function.

SUMMARY

1. The test for porphobilinogen described by Watson and Schwartz was done on the urine of 91 epileptic patients.

2. Forty-seven of these 91 patients had a definite "positive" test.

3. Studies of the chromogens, causing these positive-appearing tests failed to identify them, but did show them to be distinct from porphobilinogen aldehyde.

4. The significance of these chromogens is unknown. The suggestion is made that these chromogens may be related to a metabolic defect in epileptic patients.

I gratefully acknowledge the encouragement of Dr. R. W. Gerard and the suggestions of Dr. Leo Abood and I am particularly grateful to Dr. Herbert Grossman.

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ENDOCRINE FACTORS IN THE ALTERATIONS OF THE BLOOD COAGULATION MECHANISM FOLLOWING SURGERY

JOHN A. WILLIAMS, M.D., AND RICHARD WARREN, M.D.
BOSTON, MASS.
WITH THE TECHNICAL ASSISTANCE OF JOHN S. BELKO, M.S., AND
ALICE WYSOCKI, B.S.

EVIDENCE that the transient thrombocytopenia and depressed thrombocytogenesis characteristic of the early postoperative period[1] can be simulated by ACTH-induced "dummy stress" in normal individuals[2] suggests that adrenocortical hyperactivity may be a major factor in the altered mechanisms of blood coagulation which have been observed following surgery.[3] The potential importance of these alterations in the pathogenesis of thromboembolism prompted the present correlative evaluation of adrenocortical function and the clotting mechanism in surgical patients.

MATERIALS AND METHODS

The subjects for this study were patients on the general surgical service, undergoing elective surgical procedures of varying degrees of magnitude. None had known hematologic abnormalities. All convalesced uneventfully, without thromboembolic sequelae.

Serial observations were made pre- and postoperatively of peripheral blood platelet and eosinophil counts, coagulation times, plasma and serum prothrombin times, serum "free" 17-OH corticoid concentrations, and bone marrow megakaryocyte activities.

Blood samples were obtained by venipuncture, bone marrow by aspiration from vertebral spinous processes in the upper lumbar or lower thoracic region.

Platelets were counted by the direct method, using modified Rees-Ecker diluting fluid.[4] Absolute eosinophil counts were made by the technique of Henneman and associates.[5]

Plasma prothrombin times were determined by the method of Quick. The activity of prothrombin accessory factors was estimated by comparison of the prothrombin times of aliquots of plasma with and without the addition of material providing an excess of these factors.[6]

Residual prothrombic activity of serum was determined by the technique previously described[7]; the results are expressed in terms of the clotting time (in seconds) of the reconstituted system.

Concentrations of unconjugated 17-OH corticoids in serum were measured by the method of Nelson and Samuels.[8]

Megakaryocyte productivity was assessed by examination of stained marrow smears, utilizing a system of differential classification[1] in which megakaryocytes are grouped according to platelet content. With the exception of units whose cytoplasm is made up entirely of platelets, the percentage of platelet-containing cells is assumed to indicate productivity.

From the Surgical Service and Research Laboratory, Veterans Administration Hospital, West Roxbury, Mass., and the Department of Surgery, Harvard Medical School, Boston.

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RESULTS

As shown graphically in Figs. 1 and 2, the subject material has been considered in two groups; 10 patients subjected to relatively minor surgical procedures (such as inguinal herniorrhaphy), and 12 patients undergoing major operations (subtotal gastrectomy, pulmonary or cardiac surgery).

Group I (Major Surgery).—

Platelets: Reduction in peripheral blood platelet count during the operative procedures averaged 30 per cent. Return to preoperative range was evident on fourth postoperative day, and the ensuing thrombocytosis exceeded the preoperative mean by 45 per cent.

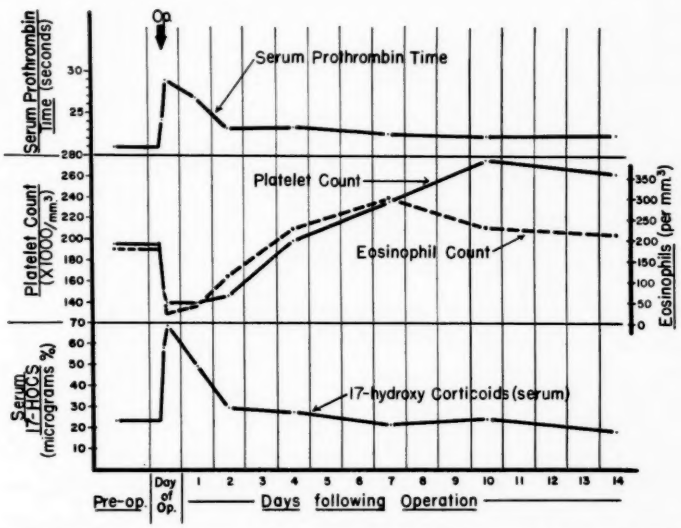

Fig. 1.—The effects of major surgery on serum corticoids, eosinophil and platelet counts, and serum prothrombin time. Mean data from 12 patients are represented.

Eosinophils: Fluctuations were similar to platelet response, except that eosinopenia was more transient and "rebound" eosinophilia occurred earlier (seventh day) in the postoperative period.

Plasma prothrombin times: One-stage prothrombin times were prolonged for ten days following operation. This trend was maximal (64 per cent of normal) on the first postoperative day and apparently represented a reduction in the activity of prothrombin accessory factors.

Megakaryocyte productivity: The ratio of platelet-producing to nonproducing cells fell from 1.15 $(=\frac{44.6}{38.8})$ before operation to 0.33 $(=\frac{10.1}{30.3})$ at the end of operation, rising to 2.0 $(=\frac{39.0}{19.7})$ on the tenth postoperative day.

Serum prothrombin times: Prompt elevation was noted, maximal at the end of operation, with normal values on and after the second postoperative day.

Serum 17-OH corticoids: Rise to highest level was attained at the end of surgery, with values in normal range on and after second day.

Group II (Minor Surgery).—The patterns of change following minor operations (Fig. 2) differ in degree rather than in kind from the responses to more major procedures.

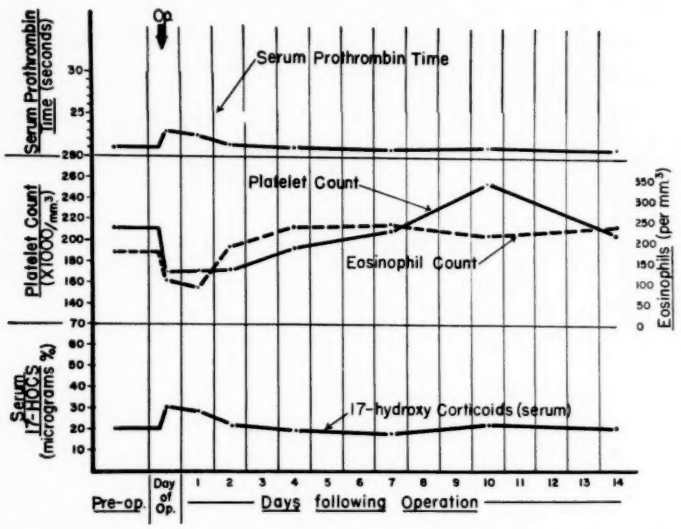

Fig. 2.—The effects of minor surgery on serum corticoids, eosinophil and platelet counts, and serum prothrombin time. Mean data from 10 patients are represented.

DISCUSSION

Acute changes occur in the clotting mechanism as well as in the peripheral level of 17-OH corticoids following surgery. The attainment of maximal corticoid levels correlates well in time with the attainment of maximal alterations in the elements of the clotting mechanism included in this report. However, with the exception of the serum prothrombin times, beyond this initial phase of the responses no direct correlation is evident between corticoids and clotting factors.

The operative rise in corticoid level was invariably accompanied by a fall in platelet count and in megakaryocyte productivity. However, in individual patients no systematic relationship was evident between the magnitudes of the corticoid response and the degrees of thrombocytopenia and depressed thrombocytogenesis. Observations not included in this report indicate, furthermore, that thrombocytopenia (and depressed platelet production) without elevation in circulating corticoids occurs occasionally after simple intravenous infusions of dextrose solutions in normal individuals. From these observations it appears unlikely that the corticoid response to surgical stress is an important factor in initiating alterations in platelet level and platelet production.

Thrombocytosis and increased megakaryocytic productivity in the second week of convalescence, found in approximately two-thirds of these patients, was not accompanied by changes in peripheral blood corticoid levels.

The correlation between the fluctuations in corticoid concentration and the efficiency of prothrombin consumption in individual patients was consistent both in timing and in magnitude. This correlation suggests that adrenocorticoids

may be the determining influence in this "superconsumption" of prothrombin, suggestive of increased activity in the thromboplastin mechanism, in the early phase of the response to stress.

SUMMARY

1. Observations were made of peripheral blood platelet and eosinophil counts, megakaryocyte productivity, plasma and serum prothrombin times, and serum 17-hydroxycorticoid concentration in patients undergoing elective surgical operations of varying degrees of magnitude.

2. Early postoperative decreases were observed in platelet and eosinophil levels, in platelet production, and in the activity of prothrombin accessory factors; these changes correlated well temporally with the observed increase in peripheral corticosteroid levels, but in each instance the alterations in these elements of the clotting mechanism persisted for several days after return of the steroid level to preoperative range.

3. Late postoperative thrombocytosis and augmented platelet production were not associated with measurable alterations in adrenocortical activity.

4. Good correlation was observed between prothrombin consumption and corticoid level throughout the periods of study.

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PLASMA AND RED CELL CHOLINESTERASE IN 800 "HEALTHY" BLOOD DONORS

J. Alfred Rider, M.D., Ph.D., J. L. Hodges, Jr., Ph.D., Joyce Swader, B.S., and Alvin D. Wiggins, M.A.
San Francisco, Calif.

THE past few years have seen a rapid increase in the number and types of anticholinesterase agents used as medications and as commercial insecticides. Great interest has also been shown in the development of anticholinesterase agents as poison gases and of effective antidotes to their use. In addition, cholinesterase activity has been utilized as an index of disease and prognosis in hepatitis and other diseases of the liver[1-11] and in ulcerative colitis.[9, 12]

Because of the growing interest in cholinesterase, it has become increasingly important to establish "normal values" for plasma and red cell cholinesterase in man. "Normal" cholinesterase levels have been reported by a number of investigators, such as Callaway and associates,[13] Sealey,[14] Fremont-Smith and associates,[10] Huerga and associates,[15] Wolfsie and associates,[16] Reinhold and associates,[17] Vorhaus and associates,[18] Kaufman,[19] Molander and associates,[20] Oka,[21] and Sabine.[22] The majority of these studies, however, were based on cholinesterase determinations by various methods in rather small groups of subjects, consisting primarily of men, in a narrow age range.

The unusual opportunity* to use blood specimens from blood-bank donors made it possible to undertake cholinesterase determinations in a large series of normal subjects with a wide age range. The purpose of this study was to establish normal values for plasma and red cell cholinesterase with respect to age and sex, to establish any variations in these values between individuals and in the same individual at different times, and to determine whether a relationship exists between plasma and red cell cholinesterase.

MATERIALS AND METHODS

Subjects.—The specimens used in this study were obtained over a 5-month period from 400 male and 400 female blood donors. The criteria of acceptance as blood donors had eliminated persons with any of the following: a history of jaundice, syphilis, tularemia, undulant or relapsing fever, diabetes, peptic ulcer, or gonorrhea; a transfusion of blood or

From the Gastrointestinal Clinic of the Department of Medicine, University of California School of Medicine, San Francisco, and the Department of Statistics, University of California, Berkeley, Calif.

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its derivatives within the past year; household contact with jaundice within one year or poliomyelitis within 3 weeks; infectious mononucleosis during the preceding 2 years, a history of rheumatic fever or tuberculosis, unless mild and of short duration; pregnancy, unless terminated 6 months previously; colds, sore throats, or infections with fever (above 99.2° F.). Persons with a temperature of 99.6° F. without other symptoms were accepted. Subjects with a history of asthma or allergy could be accepted at the discretion of the physician. A maximum pulse rate of 110 per minute and blood pressures ranging from 110/60 to 200/100 were acceptable. Serologic tests for syphilis were negative in all subjects. Hemoglobin levels in all cases were 12 Gm. or above (as determined by the copper sulfate screening method). In addition to the above criteria, no known or suspected alcoholic and no person whose appearance suggested possible malnutrition or recent disease was accepted. Less than 10 per cent of the entire group were "professional" blood donors. These are screened much more carefully than the ordinary donors, especially with regard to general health and history of present or past disease.

The donors ranged from 18 to 60 years of age. They were separated into groups according to age by year. To insure wide age spread, the groups for each year were limited to not more than 12 individuals of the same sex. Some selection inevitably occurred since the groups in the median years and the quota of men were completed first. It was more difficult to complete the groups at the extremes of the age range and to fill the quota of women.

Method.—Blood was drawn from fasting donors into 15 ml. graduated centrifuge tubes containing a small quantity of dry heparin.* The tubes were centrifuged, the plasma was removed by aspiration, and the red cells were washed twice with 0.9 per cent sodium chloride solution. The plasma and R.B.C. cholinesterase determinations were made within 24 hours, using Michel's potentiometric method[23]† which measures the fall in pH occurring when acetylcholine chloride‡ is split by cholinesterase into acetic acid and choline. The cholinesterase activity is recorded as the change in pH produced by 0.02 ml. of buffered R.B.C. or plasma with the acetylcholine substrate in one hour at 25° C., Δ pH per hour. Corrections are made for nonenzymatic hydrolysis and variations in the rate of pH change with a decrease in pH.

RESULTS

1. Accuracy of Method.—An experiment was conducted to assess the accuracy of our cholinesterase measurements and the constancy of the cholinesterase level in an individual over short periods of time. A blood sample was taken once a week for 3 weeks from each of 10 healthy subjects. Each sample was divided into 3 parts, which were analyzed separately. It was possible to estimate (1) the standard error of each analysis by comparing the triplicate analyses of the same blood sample, and (2) the standard error for the temporal variation of the cholinesterase level in the individual from week to week by comparing the mean values for the 3 blood samples and allowing for the variability of these means.

The results were as follows: *standard error of analysis,* R.B.C. cholinesterase, 0.022 ± 0.002; plasma cholinesterase, 0.022 ± 0.002; *standard error of temporal variation,* R.B.C. cholinesterase, 0.011 ± 0.004; plasma cholinesterase, 0.033 ± 0.006. These standard errors are negligible when compared with the interindividual variations.

*Supplied by The Upjohn Company, Kalamazoo, Mich.

†Although several methods are available to determine plasma and R.B.C. cholinesterase, we selected Michel's potentiometric method because it is simple, quick, highly accurate, and gives reproducible results.

‡Supplied by Merck & Co., Inc., Rahway, New Jersey.

2. The Relationship Between Plasma and R.B.C. Cholinesterase.—Although these two esterases differ, in that each has its own specific substrate, they do have in common the ability to hydrolyze acetylcholine. It is not known whether the two esterases are physiologically interdependent. A correlation has been suggested, however, in a study of ulcerative colitis,[12] where it was found that the serum cholinesterase level was low and the R.B.C. cholinesterase level high when the disease was active; whereas with healing the serum cholinesterase rose and the R.B.C. cholinesterase fell.

Analysis of the present data, however, showed little relationship between the plasma and R.B.C. cholinesterase levels in the same individual; the correlations were 0.087 ± 0.050 for men and 0.016 ± 0.050 for women. This suggests that the physiologic mechanisms that determine the two cholinesterase levels are not closely related.

TABLE I. CHANGE IN PLASMA CHOLINESTERASE LEVEL WITH AGE

MEN				WOMEN			
AGE	UPPER LIMIT	LOWER LIMIT	MEAN	AGE	UPPER LIMIT	LOWER LIMIT	MEAN
20	1.35	0.48	0.91	20	1.17	0.30	0.74
30	1.37	0.50	0.93	30	1.21	0.34	0.78
40	1.39	0.52	0.95	40	1.25	0.38	0.82
50	1.41	0.54	0.97	50	1.29	0.42	0.86
60	1.43	0.56	0.99	60	1.33	0.46	0.90

3. The Relationship of Plasma and R.B.C. Cholinesterase to the Age of the Donor.—It was found that the R.B.C. cholinesterase level did not change with age in either men or women. The plasma cholinesterase level, however, showed a definite, although small, increase with age in both sexes, amounting to 0.002 ± 0.001 Δ pH per hour per year of age in men and 0.004 ± 0.001 per year in women (Table I). This does not mean that the plasma cholinesterase levels of all individuals would necessarily increase at these rates as they grew older. For example, perhaps plasma cholinesterase levels are slightly higher in healthy than in less healthy persons, and perhaps older blood donors, as a class, are in exceptionally good health. These suppositions would explain our findings without indicating that the plasma level is actually increased with age.

4. The Relationship of Plasma Cholinesterase and R.B.C. Cholinesterase to Sex.—The levels of both cholinesterases were higher in the men than in the women donors. The difference of means was definitely significant for the plasma level (0.136 ± 0.020), but not significant for the R.B.C. level (0.016 ± 0.011).

5. The Relationship of Plasma and R.B.C. Cholinesterase to Day or Month "Batch Effects."—The samples were collected and analyzed in batches of not more than 40 a day over a period of 5 months. The variations in results from batch to batch were negligible compared to the individual variations within batches. At least 97 per cent of the total variation remains if batch differences

are entirely eliminated. Since the comparisons on the basis of age and sex were made within batches, they are not dependent on any assumption of constancy from batch to batch.

TABLE II. ESTIMATED MEANS, STANDARD DEVIATIONS, AND NORMAL RANGE OF CHOLINESTERASE LEVELS FOR AGE 40

	MEN	WOMEN
*R.B.C. Cholinesterase**		
Mean	0.766 ± 0.007	0.750 ± 0.008
Standard deviation	0.081 ± 0.008	0.082 ± 0.008
Range		
Upper limit	0.95	0.94
Lower limit	0.58	0.56
*Plasma Cholinesterase**		
Mean	0.953 ± 0.012	0.817 ± 0.017
Standard deviation	0.187 ± 0.019	0.187 ± 0.019
Range		
Upper limit	1.39	1.25
Lower limit	0.52	0.38

*Values expressed in Δ pH per hour per 0.02 ml. red cells or plasma.

6. Estimates.—The estimated means and standard deviations for plasma and R.B.C. cholinesterase in male and female donors aged 40 years are given in Table II.

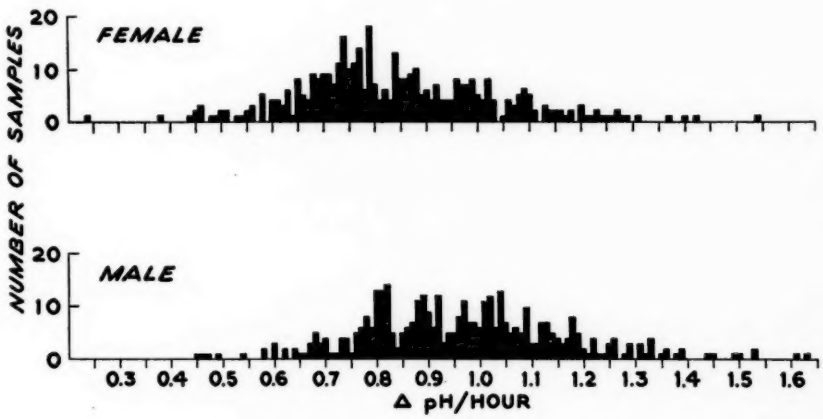

Fig. 1.—Curve showing distribution of plasma cholinesterase in 800 "healthy" blood donors (400 women and 400 men).

7. Limits of Normality.—Assuming that our blood donors are a sample of normal healthy persons and that cholinesterase levels follow the normal distribution, we can establish limits of normality of cholinesterase levels. Table II shows the normal range of R.B.C. and plasma cholinesterase levels according to sex in the donors aged 40, as calculated from our data. Ninety-eight per cent of the levels found in each group are estimated to fall between the limits shown, with 1 per cent below the lower limit and 1 per cent above the upper limit. Since the plasma but not the R.B.C. level varies slightly with age, the values for plasma cholinesterase in men should be increased about 0.02 per decade above 40 and decreased about 0.02 per decade below 40; in

women the rate of increase is 0.04 per decade. Figs. 1 and 2 show the distribution of plasma cholinesterase and of red blood cell cholinesterase in the 800 "healthy" blood donors in the present series.

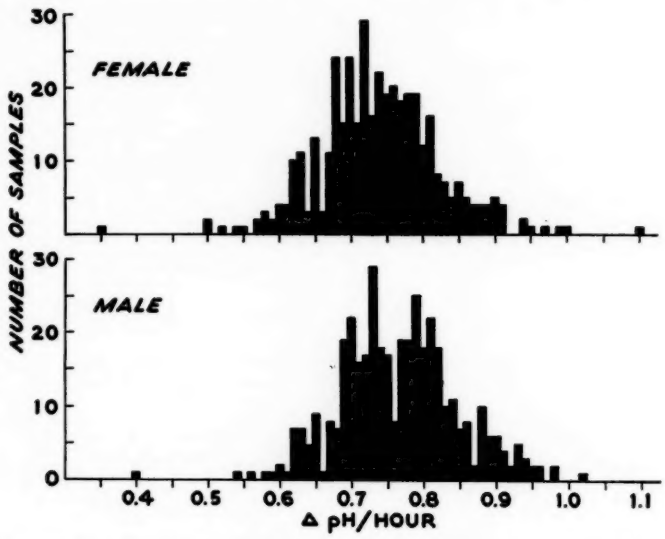

Fig. 2.—Curve showing distribution of red cell cholinesterase in 800 "healthy" blood donors (400 women and 400 men).

DISCUSSION

The earlier investigations are difficult to evaluate because of differences in methods for determining cholinesterase levels, in criteria of normalcy, and in selection of subjects. In many cases the groups tested were comprised largely of men, and little effort was made to obtain a wide age spread. In some of these studies cholinesterase levels were determined on serum rather than plasma; however, since the esterase activities of the two are identical, the data are comparable. Despite these differences, comparison of our results with those previously reported seems pertinent.

1. Results of Other Authors.—Vorhaus and Kark[18] determined the serum cholinesterase in 120 healthy individuals and found that the range of activity was 0.58 Δ pH per hour to 1.37 Δ pH per hour, with a mean of 0.94 and a standard deviation of 0.16. They concluded that differences in enzyme activity did not correlate with age, weight, sex, height, or surface area and that, although the cholinesterase activity varied widely from one healthy individual to the next, the level was "remarkably constant for each healthy, well-fed individual from day to day and from month to month."

Fremont-Smith, Volwiler, and Wood[10] found a mean serum cholinesterase activity of 0.95 Δ pH per hour, with a standard deviation of 0.24, in 20 normal men, whereas in 20 normal women the mean was 0.78, with a standard deviation of 0.12.

Wolfsie and Winter,[16] using a microadaptation of Michel's potentiometric method, tested 255 healthy male subjects and found the range for plasma

cholinesterase was 0.408 to 1.652 Δ pH per hour, with a mean of 0.912 and a standard deviation of 0.112. The range for R.B.C. cholinesterase was 0.554 to 1.252, with a mean of 0.861 and a standard deviation of 0.091 Δ pH per hour.

Sealey,[14] in a study of 67 men and women, reported a mean plasma activity of 0.92 Δ pH per hour, with a standard deviation of 0.20, and a mean R.B.C. activity of 0.773, with a standard deviation of 0.09. He stated, however, that 40 of these subjects had been minimally exposed to organic phosphate compounds.

Callaway, Davies, and Rutland,[13] using the Warburg technique, studied a group of 247 healthy adults, 66 of whom were women. They concluded that "sex, age, occupation, and season are without effect upon the level of plasma enzyme. But, in the case of the red cell enzyme, a group of servicemen were found to have a significantly higher mean level than a male civilian population of comparable age. It is conceivable that this may be due to the active outdoor life led by all of the first group, together with a high general level of physical fitness."

Reinhold, Tourigny, and Yonan,[17] using a photometric indicator method, found that the serum cholinesterase activity was higher in normal men than in women. Using a titration method, Kaufman[19] made the same observation.

2. Comparison of Our Results With Those of Other Authors.—The results of Fremont-Smith, Volwiler, and Wood[10] are in close agreement with our findings, although they did not measure R.B.C. cholinesterase.

The conclusion of Callaway et al.[13] that age has no significant effect on the R.B.C. cholinesterase level in either sex agrees with our findings. Their statement that sex and age have no effect on the level of plasma enzyme is contrary to our results. The latter conclusion may be explained by the fact that they tested a smaller number of subjects and apparently examined age dependence by graphic analysis alone, which could not be expected to detect the small increase with age found in our group. Their results also showed a somewhat higher mean cholinesterase level in men (1.87 per cent for R.B.C. and 2.6 per cent for plasma) than in women.

Our conclusion that plasma cholinesterase levels are correlated with sex and to a small extent with age are at variance with those of Vorhaus and Kark.[18] A detailed comparison cannot be made since these authors did not give the age range and sex distribution in their group. Their mean of 0.94 for serum cholinesterase is similar to our mean of 0.95 for men aged 40.

Our plasma cholinesterase values are somewhat higher and R.B.C. values somewhat lower than those reported by Wolfsie and Winter.[16] Again, since they did not give the age range of their subjects, we cannot make an accurate comparison of plasma values in the two studies. The higher R.B.C. levels found by these authors might be explained by the possibility that, since they did not wash the red cells, some plasma may have contaminated the assay.

SUMMARY

R.B.C. and plasma cholinesterase levels were determined in 800 normal, "healthy" blood-bank donors (400 men and 400 women), ranging from 18 to 60 years of age. The subjects were divided into groups according to age by year. Each group was limited to 12 individuals or less of the same sex. Blood samples were analyzed by the Michel potentiometric method in batches of not more than 40 a day over a 5-month period. The values from batch to batch were relatively constant.

Analysis of the data showed: (1) no relationship between R.B.C. and plasma cholinesterase levels; (2) a small increase in plasma cholinesterase with age; (3) no effect of age on the R.B.C. cholinesterase level; and (4) the levels of both plasma and R.B.C. cholinesterase were higher in men than in women; the difference in R.B.C. levels was not significant.

Normal cholinesterase values established by this method were as follows:

		Range (Δ pH per hour)	Mean (Δ pH per hour)
R.B.C. cholinesterase			
	Men	0.58-0.95	0.766
	Women	0.56-0.94	0.750
Plasma cholinesterase (for age 40)			
	Men	0.52-1.39	0.953
	Women	0.38-1.25	0.817

The plasma values increase slightly with age.

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THE EFFECTS OF CORTISONE ON THE GALACTOSE TOLERANCE TESTS OF NORMAL MEN

SOLOMON PAPPER, M.D., LAWRENCE SAXON, M.D.,* AND HELEN C. ALPERT, A.B.
BOSTON, MASS.

PREVIOUSLY we reported the effects of a single oral dose of 200 mg. of cortisone acetate upon intravenous fructose and glucose tolerance tests in normal men.[1] It was observed that cortisone resulted in impaired glucose tolerance but did not affect the fructose tolerance test in the same subjects.

The present study was designed primarily to determine if galactose tolerance is altered by a single large dose of cortisone. In addition, it was hoped that more information might be obtained concerning the site of cortisone action in intermediary metabolism in view of the fact that galactose enters the Embden-Meyerhof scheme at a level different from either fructose or glucose (Fig. 1).

METHODS

An intravenous galactose tolerance test was performed on two separate occasions in each of the 11 normal men receiving a regular hospital diet containing at least 250 Gm. of carbohydrate and 3,000 calories daily. In the first instance, the galactose was administered without antecedent cortisone; a few days later, the galactose was given 4 hours after the oral ingestion of 200 mg. of cortisone acetate. Immediately after an initial sample of venous blood had been obtained, the galactose was given intravenously over a 4-minute interval as a 50 per cent solution in an amount calculated to provide 0.5 Gm. galactose per kilogram of body weight. Venous blood was obtained every 15 minutes for 90 minutes after the injection and a final blood sample was drawn at 120 minutes. The experiments were begun at 8 A.M. with the subject fasting and recumbent throughout the study. Approximately 10 hours before each test each subject received 50 Gm. of glucose orally as a supplement to his regular diet.

Blood galactose was determined as follows. A 2 ml. sample of whole oxalated blood was shaken for 30 minutes at room temperature with 10 ml. of 12 per cent washed baker's yeast. Deproteinization was accomplished by the addition of zinc sulfate and barium hydroxide. Two milliliters of copper reagent were added to aliquots (0.5 to 2.0 ml.) of the blood filtrate and the tubes placed in a bath of boiling water for twenty minutes.[2] The blood obtained prior to galactose administration was similarly treated to provide a "blank" value. The determination was completed by the Nelson procedure.[3] Total blood hexose was determined by the same procedure, allowing twenty minutes for the copper reduction with omission of the yeast fermentation.[2] Galactose and glucose standards were similarly treated. The calculation of blood glucose concentration was taken as the difference between total hexose and galactose concentrations, making appropriate allowance for the different reducing powers of the two sugars as determined by their simultaneous measurement using standard solutions.

Urine was collected from the beginning of the infusion period to the end of the study and its sugar content determined by the semiquantitative method of Somogyi.[4]

From the Medical Service and the Research Laboratory, Boston Veterans Administration Hospital, and the Departments of Medicine, Boston University School of Medicine and Tufts University School of Medicine.

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*United States Public Health Service Postdoctorate Research Fellow in Medicine, Boston University School of Medicine and Boston Veterans Administration Hospital.

RESULTS

1. The galactose tolerance tests in the eleven subjects, with and without antecedent cortisone administration, are listed in Table I and the mean curves depicted in Fig. 2. With the exception of the 45-minute blood sample, there is

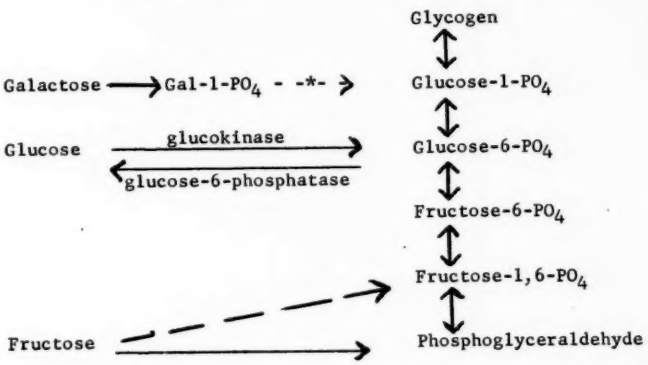

Fig. 1.—Diagrammatical representation of the site of entry of galactose, glucose, and fructose into the hepatic glycolytic cycle. *Intervening reactions in the conversion of galactose-1-PO4 to glucose-1-PO4 are omitted.

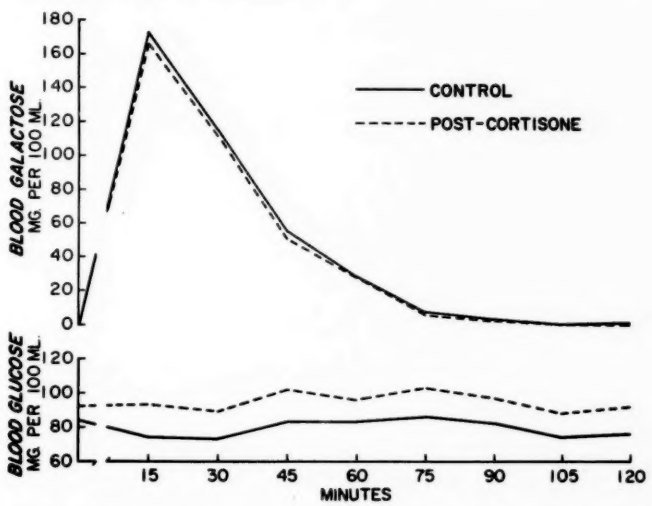

Fig. 2.—The effects of single oral dose cortisone administration on the mean galactose tolerance tests and associated blood glucose values in normal men. The time in minutes after "O" refers to the time from the end of the infusion period.

no statistically significant difference in the blood galactose levels of the control experiments and those following cortisone administration (i.e., according to the "t" test using paired comparisons P is greater than 0.1). At 45 minutes after the end of the injection, the difference between mean blood galactose levels with and without antecedent cortisone administration has a P value of < 0.01 using the same statistical methods. However, when the rate of disappearance constant (K) of galactose from the blood stream was calculated by the equation: $K = \frac{2.3 \ (\log \ C_1\text{-}C_2)}{t_2\text{-}t_1}$, in which C_1 and C_2 are the initial and final

TABLE I. THE EFFECT OF A SINGLE ORAL DOSE OF CORTISONE ON THE GALACTOSE TOLERANCE TEST

	BLOOD GALACTOSE (MG. PER 100 ML.)													
	15 MIN.*		30 MIN.		45 MIN.		60 MIN.		75 MIN.		90 MIN.		120 MIN.	
SUBJECT	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE
1	221	*213*	165	*157*	85	*83*	33	*47*	21	*12*	11	*4*	7	*1*
2	176	*178*	144	*127*	69	*57*	43	*41*	10	*7*	6	*2*	4	*0*
3	221	*203*	150	*144*	78	*74*	48	*38*	12	*6*	8	*2*	1	*0*
4	194	*168*	126	*114*	73	*60*	41	*32*	13	*9*	7	*3*	0	*2*
5	138	*145*	103	*105*	51	*46*	31	*25*	6	*12*	6	*6*	1	*4*
6	217	*216*	151	*158*	86	*83*	50	*50*	10	*12*	1	*7*	0	*2*
7	176	*168*	126	*150*	58	*61*	32	*52*	1	*2*	0	*0*	0	*0*
8	127	*100*	79	*57*	22	*11*	8	*2*	2	*3*	2	*3*	2	*0*
9	153	*160*	85	*88*	37	*35*	17	*13*	6	*7*	5	*3*	1	*3*
10	136	*120*	62	*50*	26	*13*	6	*1*	6	*0*	3	*0*	4	*0*
11	146	*154*	85	*78*	35	*36*	13	*7*	1	*0*	0	*0*	0	*0*
Mean	173.2	*165.9*	116.0	*111.6*	56.4	*50.8*	29.3	*28.0*	8.0	*6.4*	4.5	*2.7*	1.8	*1.1*
P Value	> 0.1		> 0.1		< 0.01		> 0.1		> 0.1		> 0.1		> 0.1	

*Time after completing the intravenous infusion of 50% galactose.

TABLE II. BLOOD GLUCOSE CONCENTRATION AFTER GALACTOSE INFUSION

	PRE-INFUSION		BLOOD GLUCOSE (MG. PER 100 ML.)													
			15 MIN.*		30 MIN.		45 MIN.		60 MIN.		75 MIN.		90 MIN.		120 MIN.	
SUBJECT	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE
1	86	*97*	65	*75*	53	*71*	75	*94*	92	*100*	84	*119*	80	*110*	77	*99*
2	87	*108*	77	*93*	54	*95*	80	*119*	83	*113*	96	*127*	89	*121*	86	*115*
3	82	*84*	35	*55*	50	*53*	66	*68*	66	*64*	82	*86*	79	*85*	75	*86*
4	92	*101*	93	*112*	96	*110*	106	*108*	101	*110*	109	*110*	107	*108*	89	*101*
5	78	*82*	76	*99*	66	*89*	76	*85*	73	*85*	78	*91*	77	*86*	77	*89*
6	94	*87*	56	*59*	53	*56*	72	*82*	75	*82*	90	*95*	88	*90*	76	*74*
7	83	*92*	65	*85*	69	*69*	84	*99*	76	*80*	90	*111*	80	*94*	70	*85*
8	85	*98*	94	*128*	91	*118*	101	*127*	95	*119*	86	*106*	84	*98*	80	*91*
9	73	*81*	74	*81*	88	*93*	85	*94*	82	*89*	78	*85*	73	*88*	76	*90*
10	80	*91*	87	*132*	98	*122*	88	*134*	85	*117*	74	*108*	66	*99*	60	*93*
11	79	*86*	92	*105*	87	*105*	85	*108*	82	*102*	80	*96*	77	*91*	75	*88*
Mean	83.5	*91.5*	74.0	*93.1*	73.2	*89.2*	83.5	*101.6*	82.7	*96.5*	86.1	*103.1*	81.8	*97.3*	76.5	*91.9*
P Value	< 0.01		< 0.01		< 0.01		< 0.01		< 0.01		< 0.01		< 0.01		< 0.01	

*Time after completing the intravenous infusion of 50 per cent galactose.

concentration of galactose during the period beginning t_1 minutes and ending t_2 minutes after the injection,[5] a similar statistical comparison in the interval between 15 and 45 minutes after infusion, with and without cortisone, revealed a P value greater than 0.05.

It is thus apparent that cortisone administration does not lead to impaired galactose tolerance.

2. The mean preinfusion blood glucose levels were significantly higher after cortisone than on the control day, and remained higher during the two hours following galactose administration (Table II, Fig. 2).

3. In six subjects (Subjects 1, 2, 3, 5, 6, and 7) there was a decrease in blood glucose 30 minutes after the galactose was given on the control day (Table II). In four instances (Subjects 1, 2, 3, and 6) this decrease from the fasting level exceeded 30 mg. per 100 ml. Five of these six subjects also exhibited a decrease in blood glucose following galactose infusion on the day of cortisone administration. Following cortisone, the decline was less striking than on the control day in three instances (Subjects 1, 2, and 6), more apparent in one (Subject 7), and remained unaltered in one (Subject 3). Five subjects exhibited a rise in blood glucose after the control galactose infusion varying from 4 to 18 mg. per 100 ml. This rise was increased by 14 mg. per 100 ml. or more in three subjects (Subject 8, 10, and 11) after cortisone administration and remained essentially unchanged in two (Subjects 4 and 9).

4. The urinary excretion of sugar after galactose infusion was not influenced by cortisone administration (Table III).

TABLE III. URINARY HEXOSE EXCRETION FOLLOWING THE GALACTOSE INFUSION

SUBJECT	CONTROL (GM.)	CORTISONE (GM.)
1	6.3	7.4
2	2.6	4.7
3	5.2	6.0
4	7.0	6.1
5	4.2	3.8
6	7.4	8.5
7	5.0	4.8
8	4.2	4.3
9	5.6	5.3
10	4.3	4.2
11	4.7	4.2
Mean	5.1	5.4

DISCUSSION

The precise mechanisms by which cortisone administration results in impairment of glucose tolerance remains unclear. Both increased gluconeogenesis and decreased glucose utilization have been implicated.[6-12] Our previous observations that cortisone altered glucose tolerance without affecting fructose tolerance suggested a site of action of the steroid in the metabolism of glucose. Since the pathways for glucose and fructose metabolism in the liver become identical at the triose level, cortisone must have, as one site of action, some reaction prior to the splitting of six carbon molecules into three carbon fragments in the

glycolytic cycle. Other possibilities such as a specific cortisone effect on alternate pathways of glucose metabolism (hexose monophosphate shunt) or a selective cortisone effect on glucose disposal in the periphery seemed less likely explanations.

Since galactose enters the Embden-Meyerhof scheme in the liver at a different level than either glucose or fructose (Fig. 1), the effects of cortisone on galactose tolerance may permit further definition of the site of steroid action in intermediary metabolism. Thus if galactose and glucose tolerance had been impaired without impairment of fructose tolerance, the results would have suggested that the steroid acted somewhere above the three carbon levels and below glucose-6-phosphate. To be sure, such observation could also be explained by a cortisone effect on two different sets of reactions such as the conversion of galactose to glucose-1-phosphate as well as the conversion of glucose to glucose-6-phosphate by the glucokinase reaction. On the other hand, the observation that galactose tolerance was not impaired by cortisone administration is entirely consistent with an adrenocortical steroid affect on the glucokinase reaction alone. Levine and Goldstein[13] observed that cortisone produced marked resistance to the effect of insulin on blood glucose in the intact animal, without inhibiting the action of insulin on galactose distribution after evisceration. The authors suggested that these divergent results are consistent with adrenocortical inhibition of glucokinase activity.

The physiologic significance, if any, of the lower mean 45-minute blood galactose value on the day cortisone was given remains doubtful. Hein-Sekula and Siedek[14] reported a more rapid disappearance of administered galactose from the blood after ACTH or cortisone in association with increased galactosuria. In their studies the cortisone was administered intramuscularly immediately after the galactose was given and data for individual experiments are not provided.

The decline in blood glucose after galactose administration to normal subjects has been observed before.[15, 16] It is also known that children with congenital galactosemia often have hypoglycemia.[16-18] The fact that the three subjects with blood galactose levels over 200 mg. per 100 ml. experienced striking decreases in blood glucose of more than 30 mg. per 100 ml. suggested the possibility that a high blood galactose level might stimulate insulin action or increased hepatic uptake of glucose. Such a concept is perhaps not unreasonable in view of the structural similarities of the glucose and galactose molecules, as emphasized by Levine,[19, 20] who described a similar behavior of the two hexoses in eviscerated animals in response to insulin administration. It is of interest, however, that Subject 9 who did not exhibit hypoglycemia when his blood galactose level was 153 mg. per 100 ml., did not undergo a decrease in his blood glucose level when on a subsequent occasion he received twice as much galactose and his blood galactose level exceeded 200 mg. per 100 ml.

CONCLUSIONS

1. The effects of a single oral dose of 200 mg. of cortisone acetate upon the intravenous galactose tolerance tests were studied in 11 normal subjects.

2. Cortisone did not impair galactose tolerance.

3. These observations are consistent with the hypothesis that the glucokinase reaction is a site of cortisone effect. This is not conclusively proved, nor is the possibility of other sites of action precluded.

4. Intravenous administration of galactose may result in a decrease in the blood glucose level. This observation remains unexplained.

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EFFECT OF FRUCTOSE FEEDING ON GLUCOSE TOLERANCE IN MAN

THEODORE B. VAN ITALLIE, M.D., AND KENNETH H. SHULL, PH.D.*
BOSTON, MASS.
WITH THE ASSISTANCE OF MARY B. MCCANN, M.P.H., AND HSIANG JU LIN, B.A.

HILL, Baker, and Chaikoff[1] have reported that normal rats fed an adequate diet containing fructose as the sole carbohydrate display a loss of glucose tolerance. Hill and associates ascribed the diminished capacity of the fructose-fed rat to utilize glucose to an impairment of the hepatic glucokinase system caused by protracted fructose feeding. They found that the glucose tolerance curves in fructose-fed rats resembled those observed in fasted rats; however, in fructose-fed rats the liver was the only tissue in which an altered metabolic pattern could be demonstrated in vitro.

Subsequently, Hill and Chaikoff[2] demonstrated that dogs fed a diet for one week containing fructose as the sole carbohydrate displayed an impairment of glucose tolerance similar to that observed in the fructose-fed rat. Galactose feeding did not produce such an effect in the dog.

Craig, Miller, and Drucker[3] studied human subjects who had consumed a diet containing fructose as the sole carbohydrate for 3 days. No alteration of tolerance to intravenously or orally administered glucose was observed after fructose feeding in these subjects, compared to a similar 3-day regimen in which glucose was the sole carbohydrate. Their results suggested that, unlike the rat and the dog, liver glucokinase activity in man was not decreased by a chronically lowered concentration of glucose in the portal vein blood. Van Itallie, Shull, and McCann[4] reported similar findings in human subjects fed fructose diets for 5-day periods.

In view of the conflicting results obtained in experimental animals on the one hand, and human subjects on the other, it seemed desirable to explore further the effect of fructose feeding on glucose tolerance in man. In particular, it appeared essential to obtain information about the relative roles of the liver and of the extrahepatic tissues in the disposal of ingested glucose after a period of fructose feeding. This problem was approached by use of peripheral capillary-venous glucose differences as indexes of extrahepatic glucose assimilation.

The findings indicate that, in man, fructose feeding does not lead to any impairment of net glucose tolerance. To the contrary, after ingestion of a fructose-containing diet there seems to be a significant increase in the rate at

From the Department of Nutrition, Harvard School of Public Health, Boston.

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*Postdoctoral fellow, National Heart Institute, National Institutes of Health, Bethesda, Md. Present address: Department of Biochemistry, University of Cambridge, Cambridge, England.

which peripheral tissues remove glucose from blood. Evidence obtained by indirect methods suggests that fructose feeding in man (as in the rat and the dog) may temporarily diminish the rate of glucose uptake by the liver. However, this effect is masked in the conventional glucose tolerance test by the increased peripheral glucose uptake that seems to follow ingestion for 5 days of a diet in which fructose is the sole carbohydrate.

PROCEDURE

1. *Subjects.*—Eleven graduate students without evidence of disordered carbohydrate metabolism served as subjects for 19 experiments.

2. *Experiments.*—(a) In 5 subjects, glucose tolerance after ingestion of a self-selected diet containing at least 250 Gm. of carbohydrate per day for 3 days was compared with glucose tolerance after a 5-day period during which a diet was consumed that contained fructose as the sole carbohydrate. (b) In 4 subjects, glucose tolerance was determined after 3 days of carbohydrate deprivation and compared with glucose tolerance after ingestion of a self-selected diet containing at least 250 Gm. of carbohydrate per day. (c) In 6 subjects, combined glucose-epinephrine tolerance tests after fructose feeding were compared with similar tests performed after a normal diet. Preparatory diets and procedures were the same as in 2, a. (d) In one subject, an oral fructose tolerance test was performed before and after he had remained on the fructose diet for 5 days.

3. *Diets.*—

Fructose diet: This diet furnished at least 250 Gm. of fructose* per day. Protein and fat were provided by meat and eggs. Sugar-cured meats were not used. The caloric intake was adequate to maintain body weight. The fructose was consumed as a dilute aqueous solution in small quantities evenly distributed throughout the day.

Carbohydrate-free diet: This diet was identical with the "fructose diet" except that it contained virtually no carbohydrate (less than 1.0 Gm. per day).

Normal, self-selected diet: This diet provided 250 Gm. or more of carbohydrate per day for at least 3 days before each carbohydrate tolerance test was performed.[5]

4. *Carbohydrate Tolerance Tests.*—All subjects were studied in the morning approximately 14 hours after the previous meal. In experiments 2, a and 2, b, 100 Gm. of glucose dissolved in water was administered by mouth, and capillary (finger tip) and antecubital (venous) blood sugar levels were measured at half-hour intervals for 2 hours thereafter. Control blood sugar levels were also determined. (Capillary blood contains the same concentration of glucose as arterial blood.[6])

In experiment 2, c, 30 Gm. of glucose in aqueous solution was administered by mouth 10 minutes after subcutaneous injection of 0.004 mg. of synthetic epinephrine per kilogram of body weight. Blood sugar samples were obtained immediately prior to epinephrine injection and at 30, 45, 60, 75, and 90 minutes after ingestion of the glucose.

In experiment 2, d, 50 Gm. of fructose in aqueous solution was administered by mouth, and blood fructose and glucose values and serum inorganic phosphate levels were determined at 30-minute intervals for 2 hours.

5. *Methods.*—Glucose in whole blood was measured by the method of Somogyi[7] as modified by Nelson.[8] Fructose was measured by the method of Roe.[9] Serum inorganic phosphate was measured by the method of Fiske and Subbarow.[10] Venous and capillary blood samples were collected as previously described.[11] Relative assimilation indexes (R.A.I.) were calculated for each experiment in which capillary-venous glucose differences were measured, according to Somogyi's formula.[12] (Theoretic considerations underlying this type of calculation have been discussed by Hlad, Elrick, and Witten.[13])

$$\text{R.A.I.} = \frac{100 \times \Sigma(\text{C-V})}{\Sigma(\text{i})}$$

*Kindly provided by Mead Johnson & Company, Evansville, Ind.

where, $\Sigma(C\text{-}V)$ = sum of capillary-venous differences
$\Sigma(i)$ = sum of the increments in capillary blood glucose over the control level during the corresponding time interval.

Probabilities were estimated according to Student's test of "t".[14]

RESULTS

The mean glucose tolerance curves (venous blood) in the normal subjects on (a) "normal," (b) fructose-containing, and (c) carbohydrate-free diets are shown in Fig. 1. A striking impairment in glucose tolerance is evident in the subjects who were on a carbohydrate-free diet for 3 days. When the mean glucose tolerance after 5 days of fructose feeding is compared with the mean glucose tolerance after a regular diet no such impairment is observed. If anything, glucose tolerances after fructose feeding are slightly improved as compared with those after a regular diet.

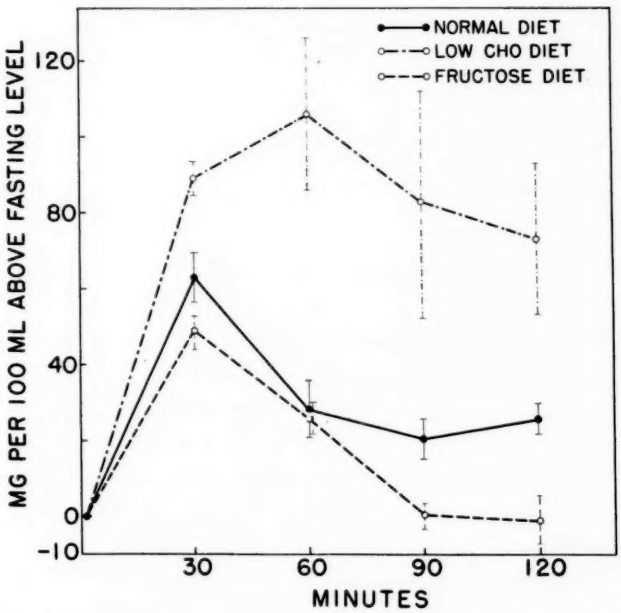

Fig. 1.—Effect of different diets on mean oral glucose tolerance (venous) of normal subjects. Vertical bars represent one standard error.

The relative (glucose) assimilation indexes (R.A.I.) calculated for the normal subjects on the 3 dietary schedules are given in Table I along with the individual capillary and venous blood sugar increments. The mean relative assimilation index of 63.3 ± 12.5* after the fructose diet is significantly greater ($p < 0.001$) than the mean relative assimilation index of 36.4 ± 6.7 after the regular diet. The mean relative assimilation index of 14.4 ± 10.3 after the carbohydrate-free diet is significantly lower ($p < 0.01$) than that after the regular diet.

*S. D. $= \sqrt{\frac{\Sigma(x-\bar{x})^2}{(n-1)}}$.

TABLE I. EFFECT OF FRUCTOSE DIET AND LOW-CARBOHYDRATE DIET ON GLUCOSE TOLERANCE IN NORMAL SUBJECTS

SUBJECT	MINUTES AFTER INGESTION OF 100 GM. GLUCOSE								RELATIVE ASSIMILATION INDEX (R.A.I.)
	30		60		90		120		
	c*	v†	c*	v†	c*	v†	c*	v†	
After Regular Diet									
T. R.	90	66	20	5	34	18	37	25	36.5
G. A.	59	45	48	29	25	3	31	16	42.9
R. C.	91	79	60	41	32	18	46	29	25.3
D. B.	91	83	68	26	27	14	28	19	32.2
R. S.	73	48	34	18	48	33	31	10	40.3
J. G.	90	59	89	50	63	39	42	20	41.1
Mean and Standard Deviation									36.4 ± 6.7
After Five Days on Fructose Diet									
T. R.	66	42	46	20	21	−3	22	−3	62.5
G. A.	78	53	39	13	23	−8	1	−25	79.4
R. C.	71	58	57	26	34	3	22	2	51.0
D. B.	59	35	59	41	32	11	34	3	51.6
R. S.	88	60	62	28	34	8	32	14	72.2
Mean and Standard Deviation									63.3 ± 12.5
After Three Days on Low-Carbohydrate Diet									
G. A.	109	80	106	73	91	70	78	46	30.4
R. C.	110	98	152	138	164	151	138	123	9.7
D. B.	122	95	87	67	65	51	39	33	20.4
J. G.	105	83	159	146	167	156	137	93	16.7
Mean and Standard Deviation									14.4 ± 10.3

*Capillary glucose increments over control value, in milligrams per 100 ml.
†Venous glucose increments over control value, in milligrams per 100 ml.

In Fig. 2 the effect of fructose feeding on the response of normal subjects to the combined epinephrine-glucose tolerance test is shown. It is apparent that after fructose feeding a marked impairment in tolerance to glucose occurred during the first 45 minutes after glucose ingestion.

The individual capillary and venous blood sugar values for the same experiment are given in Table II as well as the relative assimilation indexes calculated for each individual tolerance test. These data show that, although the mean capillary and venous blood sugar values are much higher at the 30- and 45-minute intervals after fructose feeding than after the normal diet ($p < 0.01$ and $= 0.02$), the associated mean relative assimilation indexes are not significantly different.

To exclude the possibility that fructose feeding might have increased the liver glycogen content to a degree that the hyperglycemic response to epinephrine was enhanced, the response of the blood sugar to epinephrine administration was measured in three individuals before and after five days of fructose feeding. The mean hyperglycemic response after fructose feeding for these three subjects proved to be slightly lower after fructose feeding than it was following ingestion of a regular diet (Table III).

The effect of prolonged fructose feeding on the ability of the body to convert ingested fructose rapidly to glucose was determined in one subject (Fig. 3). No change in fructose tolerance or in the blood glucose response to fructose administration was observed following consumption of the fructose-containing diet of 5 days.

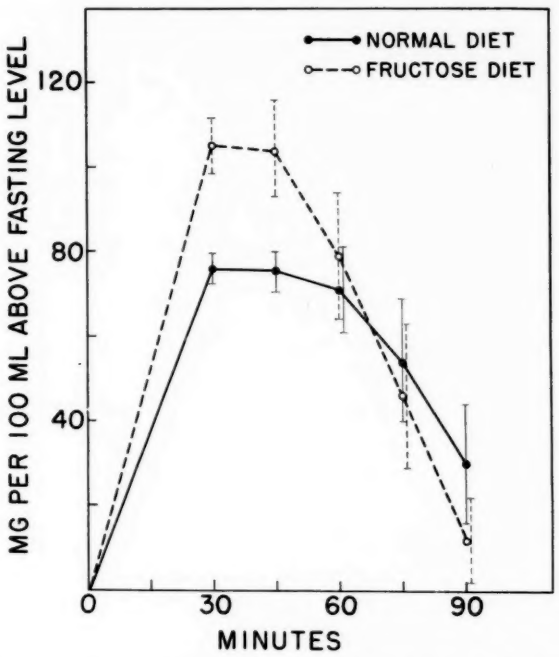

Fig. 2.—Mean hyperglycemic responses (venous) to oral glucose and subcutaneous epinephrine (glucose-epinephrine test) in normal subjects before and after fructose diet. Vertical bars represent one standard error.

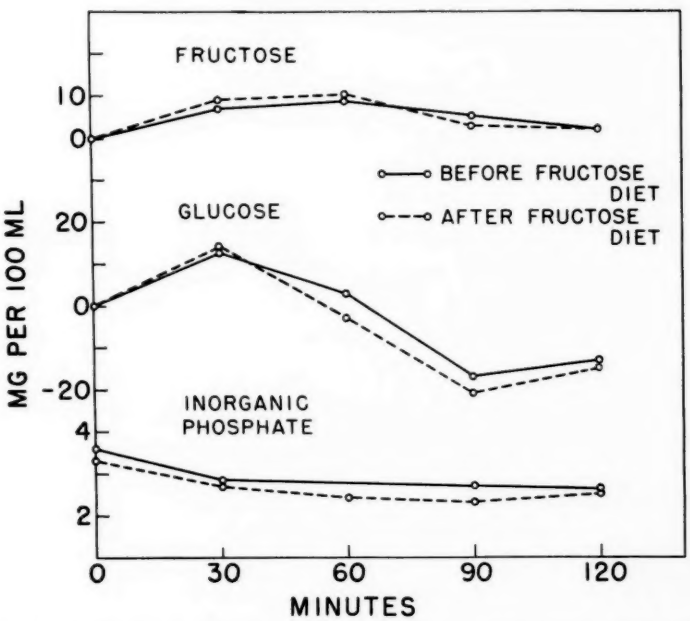

Fig. 3.—Changes in blood fructose and glucose and serum inorganic phosphate in a normal subject following ingestion of 50 Gm. of fructose, before and after a 5-day period of fructose feeding.

TABLE II. EFFECT OF FRUCTOSE FEEDING ON HYPERGLYCEMIC RESPONSE TO GLUCOSE-EPINEPHRINE ADMINISTRATION IN NORMAL SUBJECTS

SUBJECT	MINUTES AFTER INGESTION OF 30 GM. GLUCOSE										RELATIVE ASSIMILATION INDEX (R.A.I.)
	30		45		60		75		90		
	c*	v†	c*	v†	c*	v†	c*	v†	c*	v†	
After Regular Diet											
D. B.	82	69	76	66	69	65	51	44	18	15	12.5
L. B.	98	87	69	57	40	30	16	11	–6	–6	17.5
R. C.	88	86	85	79	80	75	63	60	46	43	5.2
K. M.	81	65	94	77	83	61	53	23	–	–	27.3
J. S.	73	73	91	85	101	94	95	101	–	–	3.6
T. T.‡	83	79	88	90	99	100	88	86	79	73	2.3
Mean and Standard Deviation											11.4 ± 9.7
After Five Days on Fructose Diet											
D. B.	100	77	92	73	80	68	64	47	47	37	21.1
L. B.	146	122	96	65	27	6	3	–8	–13	–19	35.9
R. C.	131	111	115	101	101	84	64	52	27	21	15.8
K. M.	125	105	137	123	118	95	–	–	–	–	9.7
J. S.	124	118	140	135	125	118	102	94	–	–	5.3
T. T.‡	102	98	136	126	126	100	–	–	–	–	11.0
Mean and Standard Deviation											16.5 ± 12.8

*Capillary glucose increments over control value, in milligrams per 100 ml.
†Venous glucose increments over control value, in milligrams per 100 ml.
‡Subject took 75 Gm. glucose by mouth.

TABLE III. MEAN RISE IN CAPILLARY AND VENOUS BLOOD GLUCOSE AFTER INJECTION OF EPINEPHRINE IN THREE NORMAL SUBJECTS BEFORE AND AFTER FRUCTOSE FEEDING

DIET	MINUTES									
	30		45		60		75		90	
	c*	v†	c*	v†	c*	v†	c*	v†	c*	v†
Normal	22	19	27	25	23	29	18	19	14	14
Fructose	19	20	20	22	16	18	16	12	9	8

*Capillary glucose increments over control value, in milligrams per 100 ml.
†Venous glucose increments over control value, in milligrams per 100 ml.

DISCUSSION

The data clearly indicate that net tolerance to glucose after 5 days of fructose feeding is not impaired in normal subjects. This is in distinct contrast to results in the rat[1] and in the dog.[2] In both of these species marked impairment of glucose tolerance has been observed after periods of fructose feeding lasting 3 and 7 days, respectively.

When glucose tolerance after fructose feeding is considered in terms of antecubital capillary-venous glucose differences, it becomes apparent that, following fructose feeding, there is an increased uptake of glucose in the forearm as compared with forearm capillary-venous differences measured during control glucose tolerance studies. The mean relative assimilation index after the fructose diet is significantly greater than that after a normal diet adequate in unrefined carbohydrate. From these observations it is inferred that fructose feeding, in the subjects studied, enhanced the rate at which the peripheral tissues removed glucose from the blood.

The enhancing effect of fructose feeding on peripheral glucose uptake was unexpected in view of the findings of Hill and associates in animals; nevertheless, the results are too striking to ignore. The mechanism of this effect of fructose feeding remains unexplained.

Net glucose tolerance after fructose feeding improved to a lesser degree than might have been predicted on the basis of the markedly increased relative assimilation indexes measured following the fructose diet.[13] This discrepancy suggested that the fructose feeding might have had an effect on the liver that was obscured by the increased rate of uptake in the peripheral tissues.

To test this possibility, a modified oral glucose tolerance test involving ingestion of a small quantity of glucose in combination with subcutaneously administered epinephrine was employed. The epinephrine was given because of its inhibitory effect on peripheral glucose uptake.[11, 12, 15, 16] It was hoped that by reducing the part played by the extrahepatic tissues in determining the rate of glucose removal from the blood, a possible effect of fructose feeding on liver uptake of glucose would be disclosed.

When the epinephrine-glucose tolerance test was done before and after the 5-day fructose regimen, a highly significant decrease in glucose tolerance was observed following the fructose diet. This decreased tolerance was not associated with a decreased relative assimilation index, in contrast to the relative assimilation indexes following carbohydrate deprivation. Since fructose is known to be readily converted to glycogen in the liver,[17] the possibility was considered that the decreased glucose tolerance after fructose feeding when the glucose-epinephrine tolerance tests were done was due merely to an enhanced hyperglycemic response to the epinephrine. However, when the hyperglycemic responses to epinephrine alone before and after fructose feeding were compared, they did not differ (Table III).

It is difficult to understand why changes in net glucose tolerance after fructose feeding of the kind reported for the rat and the dog have not been demonstrable in human subjects. The proportion of liver weight to total body weight is approximately the same in all three species; hence, it does not seem possible to account for the observed differences by reference to relative liver size. It is recognized that rat, dog, and man differ somewhat in their characteristics of carbohydrate metabolism; however, not enough is known about the relative roles of liver and extrahepatic tissues in glucose disposal in the three species to explain why man should fail to show the same impairment of over-all glucose tolerance after a fructose diet as the rat and the dog.

It is assumed that ingestion of single doses of fructose is not associated with appreciable changes in blood glucose in nondiabetic individuals. However, the suggestion has been made that prolonged fructose feeding is associated with an increased ability of the body to convert fructose to glucose so that the feeding of fructose will be associated in due course with considerable rises in blood glucose.[18] Accordingly, the effect of 5 days of fructose feeding on the blood glucose response to a large quantity of ingested fructose was determined. No changes in the blood fructose and glucose curves after ingestion of 50 Gm. of

fructose took place as a result of the 5-day fructose diet. In both instances, the blood glucose level rose slightly at first and then fell below postabsorptive values following fructose ingestion. Similar responses of blood glucose to fructose administration have been reported by Miller and associates[19] and by Pletscher, Fahrländer, and Staub.[20]

The impaired glucose tolerance curves and decreased relative assimilation indexes associated with carbohydrate deprivation are consistent with the findings of other investigators suggesting that the "Hofmeister" effect is due principally to inhibition of glucose uptake in the periphery.[21] It should be pointed out that while fructose feeding and carbohydrate restriction both might be expected to reduce insulin requirements materially, fructose feeding in adequate amounts would not be associated with any deficiency of liver glycogen.[17] In contrast, acute carbohydrate deprivation leads to pronounced diminution of hepatic glycogen stores.[21] Thus, it may be that hepatic glycogen depletion is the sine qua non of starvation diabetes. If this difference in liver glycogen status is kept in mind, it is not surprising that the effect of fructose feeding on carbohydrate tolerance is entirely different from that of carbohydrate restriction. If anything, fructose feeding is associated with an increased rather than a decreased rate of glucose uptake in the extrahepatic area.

SUMMARY

The effect on glucose tolerance of an adequate diet containing fructose as the sole carbohydrate was determined in a series of normal subjects. Information about the relative roles of liver and extrahepatic tissues in the disposal of ingested glucose after a period of fructose feeding was obtained by use of peripheral capillary-venous glucose differences as indexes of rate of extraction of glucose by forearm tissues.

Although over-all glucose tolerance and rate of uptake of glucose by the forearm tissues were markedly diminished following ingestion of a carbohydrate-free diet for 3 days, the 5-day fructose diet did not induce any impairment of net tolerance to glucose or in rate of peripheral glucose uptake. Indeed, the rate of peripheral glucose removal was significantly increased following ingestion of the fructose diet.

The effect of fructose feeding upon glucose tolerance was also determined in subjects who had been given a small quantity of epinephrine subcutaneously just prior to the glucose tolerance tests, in an attempt to reduce the influence of the extrahepatic tissues upon rate of glucose removal from the blood. When epinephrine-glucose tolerance tests before and after the 5-day fructose regimen were compared, a significant decrease in net glucose tolerance was observed following the fructose diet. The decreased tolerance was not associated with a decreased rate of peripheral glucose assimilation, as measured by capillary-venous glucose differences, and this result suggested that fructose feeding may have impaired temporarily the ability of the liver to remove glucose from the circulation. Hyperglycemic responses to epinephrine alone measured before and after comparable periods of fructose feeding did not differ significantly.

It is concluded that the impaired glucose tolerance associated with carbohydrate deprivation is due principally to a decreased uptake of glucose in the extrahepatic tissues, and that the effect of fructose feeding on glucose tolerance is entirely different from that of carbohydrate restriction.

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THE ACUTE RESPIRATORY EFFECTS OF CHLORPROMAZINE IN MAN

ATTILIO D. RENZETTI, JR., M.D., AND WILLIAM R. PADGET, M.D.
SYRACUSE, N. Y.

CHLORPROMAZINE is being used extensively in the practice of medicine and psychiatry and as a preanesthetic medication. Because of this widespread use, it seemed desirable to have a quantitative estimation of the respiratory action of this drug in man and thereby an evaluation of one of its potential dangers. Dobkin and his associates,[1] from a study of both anesthetized and unanesthetized subjects, concluded that chlorpromazine caused central depression of respiration. Using a closed circuit apparatus, such as those in common use for the estimation of basal metabolism rate, these authors found that after intravenous administration of the drug the tidal volume was consistently decreased, but arterial blood O_2 and CO_2 contents were unchanged. Since a CO_2 absorber was in the system, no measurement of CO_2 output was made. Tenney[2] has emphasized that for accurate interpretation of drug effects on the respiratory mechanisms, a measure of alveolar ventilation, arterial CO_2 tension, and CO_2 output must be available.

The purpose of this communication is to report the effects observed in normal individuals and emphysematous patients after the administration of chlorpromazine.

SUBJECTS FOR STUDY

The age, physical characteristics, and diagnosis of each of the patients studied are recorded in Table I. Subjects 1 through 7 were patients admitted for a scheduled surgical procedure and by history, physical examination, and chest x-ray were found to be normal except for the defect listed under diagnosis. These individuals are referred to below as normals. Subjects 8 and 9 had both had previous clinical and physiologic study with findings characteristic of chronic pulmonary emphysema. Subject 8 had recently recovered from right heart failure and CO_2 narcosis.

TABLE I. PHYSICAL CHARACTERISTICS AND DIAGNOSIS OF PATIENTS GIVEN CHLORPROMAZINE

PATIENT	AGE (YR.)	HEIGHT (CM.)	WEIGHT (KG.)	BSA (SQ. METER)	DIAGNOSIS
1. K. B.	33	178	80	1.98	Ventral hernia
2. L. R.	32	170	76	1.86	Pilonidal sinus
3. C. J.	45	176	66	1.80	Varicose veins
4. H. H.	30	178	74	1.92	Varicose veins
5. P. R.	49	174	77	1.92	Hemorrhoids
6. B. P.	37	173	73	1.86	Inguinal hernia
7. A. P.	32	179	79	1.98	Pilonidal sinus
8. L. G.	59	163	62	1.68	Emphysema
9. L. D.	65	166	68	1.76	Emphysema

From the Chest Disease Division and Cardio-Pulmonary Laboratory of the Veterans Administration Hospital, and the Department of Medicine, State University of New York College of Medicine, Syracuse, N. Y.

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METHODS

Each patient was brought to the laboratory after an overnight fast and all observations were made while the individual was in a comfortable supine position. An indwelling arterial needle was placed in the brachial artery and compressed air or 5 per cent CO_2 in air was administered through an open circuit using an anesthesia bag, mouthpiece, and a valve with a dead space of approximately 60 ml. Expired air was collected and measured in a Tissot spirometer. For each experiment, expired gas was collected for 3 minutes and arterial blood was sampled anaerobically during the middle minute. The composition of inspired and expired gas was analyzed on the Scholander gas analyzer.[3] The O_2 content and capacity and CO_2 content of the blood were determined by the method of Van Slyke and Neill.[4] The O_2 and CO_2 tensions were measured by the technique of Riley, Proemmel, and Franke.[5] In Subjects 3, 8, and 9, the CO_2 tensions recorded are those obtained by use of the Van Slyke and Sendroy charts[6] after whole blood pH had been determined on a Cambridge research model pH meter. Five per cent CO_2 in air was inspired prior to the 3-minute observation period for 12 minutes by Subjects 5 and 6, and for 25 minutes by Subjects 8 and 9. Blood pressure was measured by a sphygmomanometer.

Volumes of ventilation were corrected to body temperature and pressure, saturated with water vapor (BTPS), whereas quantities of gas exchanged are reported at zero degrees centigrade, 760 mm. Hg pressure and dry (STPD). The respiratory dead space was calculated by use of the Bohr equation, utilizing the assumption that arterial is equal to alveolar CO_2 tension. Alveolar ventilation was then calculated according to the formula: alveolar ventilation = minute volume expired − (percentage dead space × minute volume expired).

The chlorpromazine used in these studies was Thorazine, 25 mg. per cubic centimeter and the total dose administered was 25 mg. intravenously or 50 mg. intramuscularly. When the intravenous route was used, the injection period was 5 minutes.

RESULTS

The intravenous injection of chlorpromazine was followed in 15 to 30 minutes by a drowsy or sleeping state from which the patients could easily be aroused. Only mild drowsiness occurred in one (Patient 8) who received the drug intramuscularly. The results of measurements of ventilation, arterial blood gas composition, pulmonary gas exchange, blood pressure, and pulse are summarized in Tables II and III.

A. *Ventilatory Effects.*—Following the intravenous administration of 0.32 to 0.38 mg. per kilogram of chlorpromazine to 6 normal individuals (Subjects 1 to 6), there occurred only small decreases in total minute ventilation in 5 and in average tidal volume in 4. Respiratory frequency also showed small and variable changes except in Patient 2 whose rate decreased markedly. However, it may be seen from the representative respiratory tracings reproduced in Figs. 1 and 2 that irregularities of rhythm and tidal volume occurred. These tracings are a record of the manner in which the Tissot spirometer was filled with expired air during the 3-minute periods. Since spirometer volume was constant in inspiration, no record of the ventilatory pattern for this phase of respiration is shown. Also, since the paper speed is slow, the record gives only a gross measure of the rate of air flow during expiration. Nevertheless, the general pattern observed was one of regular shallow breaths with intermittent deep sighing ones. This abnormality of ventilatory pattern led to an increase in physiologic dead space in all of these individuals and hence all

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TABLE II. EFFECT OF CHLORPROMAZINE ON VENTILATION, ARTERIAL BLOOD GAS, AND PULMONARY GAS EXCHANGE

PATIENT	TIME (MIN.)	INSPIRED GAS	$\dot{V}_E$ (L./MIN.)	FREQ. RES.	V_T (C.C.)	Pa_{O_2} (MM. Hg)	Sa_{O_2} (%)	Pa_{CO_2} (MM. Hg)	$\dot{V}_{O_2}$ (C.C./MIN.)	$\dot{V}_{CO_2}$ (C.C./MIN.)	RES. QUOT.
					A. Normal Individuals						
1. K. B.	−6*	Air	9.31	19	490	75	95	39	261	221	0.85
	0	0.32 mg. per kilogram chlorpromazine intravenously									
	+15	Air	6.80	17	400	74	93	40	263	153	0.58
	+45	Air	7.72	19	406	74	94	43	238	169	0.71
2. L. R.	−6*	Air	7.26	18.3	397	85	95	45	236	193	0.82
	0	0.33 mg. per kilogram chlorpromazine intravenously									
	+20	Air	5.93	9	659	79	94	54	223	163	0.73
	+40	Air	6.78	14.3	474	89	95	58	219	190	0.87
	+60	Air	6.85	16.3	420	93	95	47	200	172	0.86
3. C. J.	−6	Air	8.58	12	715	98	99	33	274	239	0.84
	0	0.38 mg. per kilogram chlorpromazine intravenously									
	+10	Air	8.41	16	526	--	95	38	270	194	0.72
	+20	Air	6.94	18.7	371	81	92	40	205	114	0.56
4. H. H.	−6*	Air	9.68	20	484	--	95	36	254	216	0.85
	0	0.34 mg. per kilogram chlorpromazine intravenously									
	+15	Air	10.67	21.3	501	--	94	38	246	214	0.87
	+30	Air	10.25	20	513	--	94	43	240	212	0.88
5. P. R.	−6*	Air	6.55	12.3	533	89	96	37	237	183	0.77
	0	0.33 mg. per kilogram chlorpromazine intravenously									
	+11	Air	6.26	14.7	426	--	96	37	207	152	0.73
	+18	Air	7.14	14	510	--	92	46	181	154	0.85
	+53	5% CO_2 in air	21.6	16	1,350	118	98	64	206	156	0.76

6. B. P.	−6*	Air	9.14	14.7	622	90	93	35	276	249	0.90
	0	0.35 mg. per kilogram chlorpromazine intravenously									
	+10†	Air	9.26	16	579	81	92	37	284	245	0.86
	+30	Air	7.46	16.3	458	--	92	37	235	211	0.90
	+62	5% CO_2 in air	20.10	16.3	1,233	124	98	48	274	137	0.50
7. A. P.	−6*	Air	7.64	16.7	457	95	96	40	261	216	0.83
	0	0.63 mg. per kilogram chlorpromazine intramuscularly									
	+30	Air	7.79	15.7	496	--	96	37	251	215	0.86
	+60	Air	7.89	17.3	456	99	94	37	245	215	0.88
		B. Patients With Emphysema									
8. L. G.	−44	Air	8.28	21.7	382	39	68	59	210	163	0.78
	−3	5% CO_2 in air	11.39	21	542	66	89	74	234	165	0.71
	0	0.8 mg. per kilogram chlorpromazine intramuscularly									
	+21	Air	7.76	17.3	449	54	75	66	196	170	0.87
	+46	5% CO_2 in air	12.74	17.3	736	71	92	76	219	161	0.74
9. L. D.	−44*	Air	9.17	14.7	381	74	94	50	215	176	0.82
	−3	5% CO_2 in air	12.45	18	374	102	98	62	225	170	0.76
	0	0.69 mg. per kilogram chlorpromazine intramuscularly									
	+60	Air	7.16	11	365	59	87	52	210	183	0.87
	+85	5% CO_2 in air	13.61	14.3	476	104	97	60	216	185	0.86

*Indicates two control studies.

†Patient developed shock with unobtainable blood pressure and pulse at 15 minutes. Responded to 2 c.c. of Neosynephrine intravenously.

Key to abbreviations: $\dot{V}_E$ = expired ventilation; V_T = tidal volume; Freq. Res. = frequency of respirations per minute; PaO_2 = arterial oxygen tension; SaO_2 = arterial oxygen saturation; $PaCO_2$ = arterial carbon dioxide tension; $\dot{V}O_2$ = oxygen uptake; $\dot{V}_{CO2}$ = carbon dioxide output; Res. Quot. = respiratory quotient.

Fig. 1.

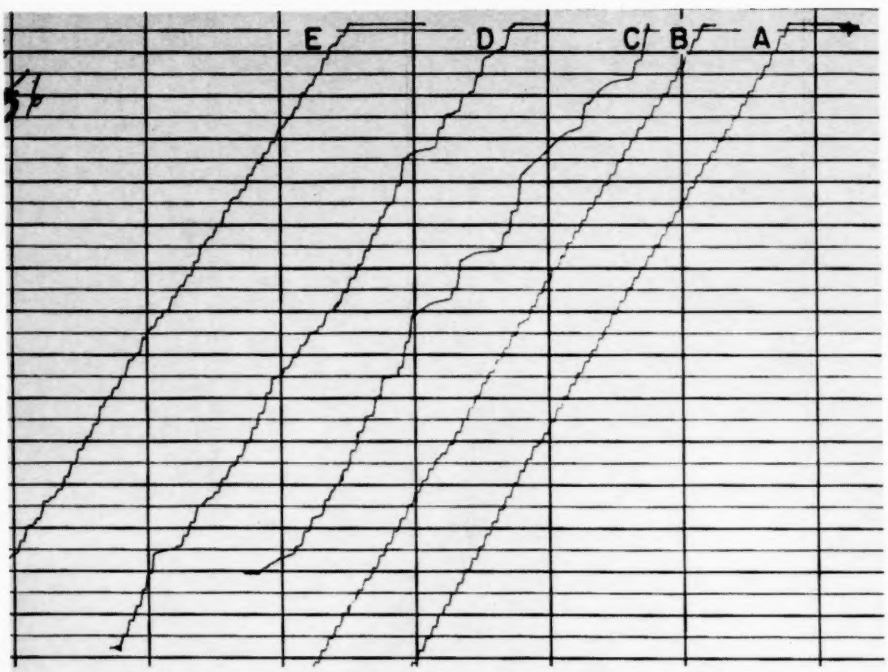

Fig. 2.

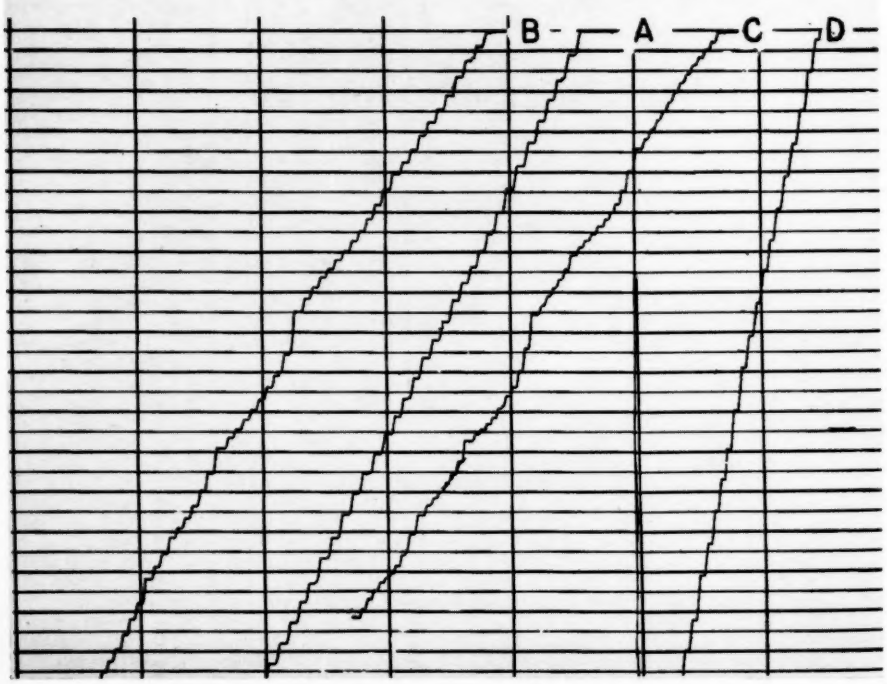

Fig. 1.—Spirometer tracing of respiratory excursions (Subject 2, L.R.). A and B = control. C = 20 minutes, D = 40 minutes, E = 60 minutes after I.V. chlorpromazine. Time = 1 minute between each vertical line. Inspiration = exactly horizontal since the spirometer volume is constant. Expiration = all lines with vertical component.

Fig. 2.—Spirometer tracing of respiratory excursions. A, B, and C = control, 10 and 20 minutes respectively, after I.V. chlorpromazine for Subject 3 (C.J.). D = inhalation of 5 per cent CO_2 62 minutes after I.V. chlorpromazine for Subject 6 (B.P.). See legend to Fig. 1.

showed a decrease in alveolar ventilation. These effects were at their maximum between 15 and 30 minutes after injection of the drug but were still present at the end of 60 minutes. They did not occur after a 0.63 mg. per kilogram dose given intramuscularly in a normal individual.

B. *Effects on Blood Gas Composition and Pulmonary Gas Exchange.*— Chlorpromazine given intravenously resulted in an elevation of arterial carbon dioxide tension of 6 mm. or more in 4 individuals (Subjects 2 through 5)

TABLE III. EFFECT OF CHLORPROMAZINE ON RESPIRATORY DEAD SPACE, ALVEOLAR VENTILATION, BLOOD PRESSURE AND PULSE

PATIENT	TIME (MIN.)	INSPIRED GAS	V_D/V_T (%)	$\dot{V}_A$ (L./MIN.)	B.P. (MM. HG)	PULSE
			A. Normal Individuals			
1. K. B.	−6*	Air	46	5.03	94/58	60
	0	0.32 mg. per kilogram chlorpromazine intravenously				
	+15	Air	50	3.40	98/54	80
	+45	Air	55	3.46	90/50	70
2. L. R.	−6*	Air	48	3.77	100/60	72
	0	0.33 mg. per kilogram chlorpromazine intravenously				
	+20	Air	55	2.66	100/60	76
	+40	Air	56	2.98	100/60	80
	+60	Air	53	3.22	80/40	72
3. C. J.	−6	Air	40	5.14	132/70	84
	0	0.38 mg. per kilogram chlorpromazine intravenously				
	+10	Air	46	4.55	120/70	100
	+20	Air	63	2.56	86/?	100
4. H. H.	−6*	Air	45	5.32	100/60	68
	0	0.34 mg. per kilogram chlorpromazine intravenously				
	+15	Air	53	5.01	100/64	80
	+30	Air	57	4.42	100/60	80
5. P. R.	−6*	Air	36	4.19	124/68	76
	0	0.33 mg. per kilogram chlorpromazine intravenously				
	+11	Air	42	3.63	96/60	98
	+18	Air	58	2.99	75/56	60
	+53	5% CO_2 in air	77	4.97	100/60	90
6. B. P.	−6*	Air	30	6.40	114/64	70
	0	0.35 mg. per kilogram chlorpromazine intravenously				
	+10†	Air	37	5.84	108/60	80
	+30	Air	33	5.04	90/0	80
	+60	5% CO_2 in air	47	10.7	120/60	80
7. A. P.	−6*	Air	45	4.20	122/70	110
	0	0.63 mg. per kilogram chlorpromazine intramuscularly				
	+30	Air	34	5.14	122/70	110
	+60	Air	35	5.13	112/68	110
			B. Patients With Emphysema			
8. L. G.	−44*	Air	73	2.24	130/80	80
	−3	5% CO_2 in air	66	3.87		
	0	0.8 mg. per kilogram chlorpromazine intramuscularly				
	+21	Air	70	2.33	130/80	80
	+46	5% CO_2 in air	72	3.57		
9. L. D.	−44*	Air	61	3.58	130/80	80
	−3	5% CO_2 in air	54	5.73		
	0	0.69 mg. per kilogram chlorpromazine intramuscularly				
	+60	Air	56	3.15	130/80	80
	+85	5% CO_2 in air	50	6.81		

*Indicates two control studies.

†See footnote to Table II.

Key to abbreviations: V_D = physiological dead space; V_T = Tidal Volume; $\dot{V}_A$ = Alveolar Ventilation; B.P. = Blood Pressure.

and 4 mm. in one (Subject 1). The remaining individual (Subject 6) showed no significant change in this respect but his study was modified by the administration of Neosynephrine for the treatment of shock. Except for mild decreases in arterial oxygen hemoglobin saturation in 2 patients (Subjects 3 and 5), the changes in this value were within the limits of analytic error. However, the general trend for arterial oxygen saturation to fall following the drug was confirmed in two instances (Subjects 3 and 6) by measurements of oxygen tension. Except for one person who showed only a slight fall in CO_2 output, all showed a significant decrease in this respect as well as in oxygen uptake. The respiratory quotient also decreased in 5 of these 6 individuals although the degree of change varied widely. It should be pointed out in this respect that the achievement of a steady state following intravenous drug administration is not to be expected since drug concentration in the blood is presumably changing with time. In the normal person who received the medication intramuscularly, no significant changes in blood gas or gas exchange were observed.

C. *Illustration of Gas Exchange Effects.*—Tenney[2] has pointed out the usefulness of a graph such as Fig. 3 in the understanding of the respiratory action of drugs. In such a graph, where the abscissa represents arterial CO_2 tension and the ordinate relative CO_2 production, one can plot isopleths which represent alveolar ventilation ratios. The control CO_2 production is arbitrarily made equal to 1 and subsequent observations are related to it so that a fall in CO_2 production by one-half in the course of an experiment would be indicated by 0.5 on the ordinate. Movement of an arterial point horizontally following drug administration indicates a pure respiratory action whereas vertical movement indicates a pure metabolic action. The effect of intravenously administered chlorpromazine to the 6 normal patients in this study is shown by line A. The control point of this line is defined by the average of the 6 control arterial CO_2 tensions at a relative CO_2 production of 1. The final point represents the average relative CO_2 production and arterial CO_2 tension at the point of maximum change after drug injection. It is evident from this line that chlorpromazine has both a respiratory and metabolic depressant action.

D. *Effect of CO_2 Stimulation.*—The inhalation of CO_2 after chlorpromazine led to a restoration of a regular respiratory rhythm in all 4 patients who received it. The increase in tidal volume in the 2 normal individuals was to a degree expected in normal people who have not received drugs.[7] The ventilatory response to CO_2 stimulation is illustrated graphically in Fig. 4 where the arterial CO_2 tension is plotted on the abscissa and the alveolar ventilation ratio on the ordinate. The alveolar ventilation ratio is defined as the alveolar ventilation obtained during CO_2 stimulation divided by the alveolar ventilation observed in the control period. The expected increase in alveolar ventilation ratio for a given rise in arterial CO_2 tension in a normal individual is described by line A in Fig. 3 and mathematically by the equation: alveolar ventilation ratio $= 0.33\ pCO_2 - 11$.[8] The response of Subjects 5 and 6 to CO_2

stimulation after intravenous chlorpromazine is shown by lines B and C, respectively, and is obviously less than expected. In a patient with emphysema and elevated arterial pCO_2, a control CO_2 stimulation must be obtained if valid conclusions are to be drawn. Hence, lines D and E in Fig. 3 are the plotted responses to CO_2 stimulation, respectively, before and 46 minutes after intramuscular chlorpromazine in Subject 8. They indicate the decreased sensitivity of this patient's respiratory center and the further decrease which resulted from the drug. At the 85-minute observation point this drug effect could not be demonstrated in the other emphysematous patient (Subject 9).

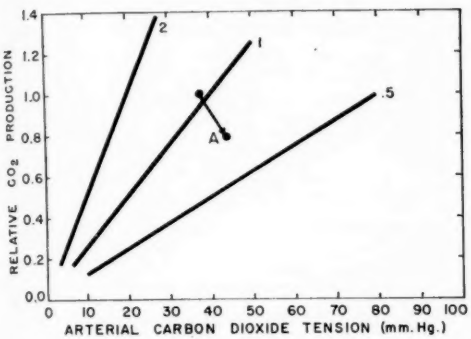

Fig. 3.

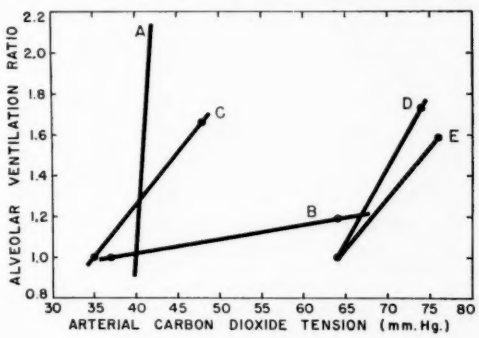

Fig. 4.

Fig. 3.—Interrelationships of CO_2 production, arterial CO_2 tension and alveolar ventilation. A = line joining average control point of 6 normal individuals and average point 15 to 30 minutes after intravenus chlorpromazine. Straight lines represent alveolar ventilation ratio isopleths with the indicated values.

Fig. 4.—Stimulus response lines to CO_2 inhalation. A = normal; B = normal subject (P.R.) 53 minutes after I.V. chlorpromazine; C = normal subject (B.P.) 62 minutes after I.V. chlorpromazine; D = emphysematous subject (L.G.) control; E = emphysematous subject (L.G.) 46 minutes after I.M. chlorpromazine.

E. *Effect on Blood Pressure.*—The arterial blood pressure decreased to a variable degree in 5 of the 6 patients who received the drug intravenously but in none of those who received it intramuscularly. Subject 6 is of particular interest in that his blood pressure fell to unobtainable levels about 15 minutes after drug administration. Concomitant with this fall, the patient complained of nausea and then of feeling faint, and he developed marked pallor. One cubic centimeter of Neosynephrine intravenously led to immediate clinical improvement and a rise in blood pressure which lasted about 5 minutes. It was only after a second intravenous dose of Neosynephrine that blood pressure remained at a normal level.

DISCUSSION

From the observations reported here, it is evident that the intravenous administration of chlorpromazine results in an abnormal ventilatory pattern characterized by regular shallow breaths interrupted irregularly by deep sighing respirations and simulating that found in normal sleep. The intermittent large tidal volumes suggest that the force of muscular contraction as well as neuromuscular transmission were not impaired by the drug. In view of the other central nervous system actions of this medication it seems likely that

its respiratory action is also a central depression. The type of respiration observed here was like that described by Hoff and Breckenridge[9] as one of the basic medullary rhythms which appears when the medulla and lower pons are released from suppressor impulses arising in the upper brain stem or cortex by decerebration or by narcotics and sleep. According to these authors, the medullary respiratory center has an intrinsic autorhythmicity which is modified by facilitatory and suppressor influences from numerous central and peripheral sources. The inhalation of 5 per cent CO_2 after chlorpromazine depression reesulted in a restoration of a regular rhythm and a normal increase in tidal volume yet the total ventilatory response was reduced. It thus seems possible that the drug has a depressant action upon the medullary respiratory center as well as more rostral areas of the brain.

Although the magnitude of the changes in blood gas tensions and contents were not great following the use of chlorpromazine, it should be pointed out that the techniques used in this study tend to obscure the greater deviations from normal that probably occurred. Since arterial blood was sampled during a one-minute period, the O_2 and CO_2 contents found represent a physiologic average of the net effects of many respiratory and cardiac cycles. In like manner, the values for alveolar ventilation, being derived from the respiratory volume and frequency of a 3-minute period, are also averages. Riley and Cournand[10] have pointed out that variations in the partial pressure of O_2 and CO_2 in alveolar gas and alveolar capillary blood from moment to moment must occur and that such variations during the respiratory cycle are likely to have an appreciable effect upon the physiologic dead space and venous admixture, particularly when the breathing is irregular. Although the calculated net effect of chlorpromazine was a reduction in alveolar ventilation, it seems likely in view of the irregularity of ventilatory depth that variations in ventilation-perfusion ratios with respect to time occurred and contributed to the gas exchange defects.

From the standpoint of therapeutic usage, these respiratory effects of chlorpromazine and the occurrence of shock in one patient emphasize the potential hazard of the intravenous route of administration. In addition, caution and close observation would seem to be advisable when this drug is administered by any route to patients who are to receive further central nervous system depressants or to individuals whose respiratory center is already depressed, such as patients with chronic pulmonary emphysema and CO_2 retention.

Diminished O_2 consumption following the use of chlorpromazine has been observed previously, both in vitro and in vivo, in animals and in man.[11] The mechanism of this action has not been elucidated.

SUMMARY AND CONCLUSIONS

The acute effects of chlorpromazine on ventilation, blood gas composition, and pulmonary gas exchange have been measured in 7 normal subjects and 2 patients with chronic pulmonary emphysema and CO^2 retention. The results

demonstrated were an irregularity of rhythm and amplitude of ventilation, alveolar hypoventilation, a decrease in O_2 uptake and CO_2 output and a diminished sensitivity of the respiratory mechanism to CO_2 inhalation. These observations indicate that chlorpromazine has a respiratory depressant action.

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THE INFLUENCE OF STREPTOCOCCAL TOXIN ON THE COURSE OF CANINE EXPERIMENTAL NEPHROSIS

KHALIL G. WAKIM, M.D.,* AND BERNARD F. MCKENZIE, M.S.**
ROCHESTER, MINN.

IN A previous communication we reported on the production of experimental nephrosis in dogs by intravenous administration of serum from rabbits sensitized to dog kidney.[1] The renal disease so produced was characterized by hypoproteinemia, with reduction of serum albumin, alpha-1 globulin and gamma globulin, and increase in alpha-2 and beta globulin. Hyperlipemia, elevation of blood urea, proteinuria, and some signs of fluid retention were among the salient features of the disease. After a certain period the animals appeared completely recovered and their blood and urinary constituents returned to normal.

In patients suffering from the nephrotic syndrome the clinical history not infrequently reveals association of the disease with septic sore throat. Since the experimental renal disease so closely resembles in its salient features the clinical condition in man designated by the term "nephrotic syndrome," we decided to investigate in animals the influence of administration of streptococcal toxin on the course of the nephrotic state induced in dogs by administration of nephrotoxic serum from rabbits sensitized to dog kidney. A number of investigators[2-6] have produced this renal disease in rats, rabbits, and occasionally puppies with some minor variations in the different species. We found the dog to be a very satisfactory animal for the experimental production of this syndrome.

METHODS

In the present study we used young immunized dogs in which the serum proteins, urinary proteins, serum cholesterol, and blood urea were quantitated before and after the administration of the nephrotoxic agents and their concentrations were followed until the animals recovered.

Healthy dogs were divided into three groups. One group was given nephrotoxic serum prepared, according to the procedure previously described,[1] from rabbits which had been sensitized against dog kidney. Exsanguination of the rabbits was done by cardiac puncture, which yielded a larger volume of blood than the previous procedure of cannulation of the femoral artery. A second group of dogs was given a streptococcal culture filtrate. This filtrate was prepared by filtering a 4-day culture in veal infusion broth of *Streptococcus pyogenes* (C203M) through a Mandler candle.† A third group was given a mixture of nephrotoxic serum and streptococcal culture filtrate. The total dose of each of the

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*Section of Physiology, Mayo Clinic and Mayo Foundation.
**Section of Biochemistry, Mayo Clinic and Mayo Foundation.
†Dr. F. R. Heilman prepared the filtrate for this study.

nephrotoxic serum and of the streptococcal culture filtrate was 4.4 ml. per kilogram of body weight divided into 9 doses and given 3 times daily over 3 days. In the dogs receiving the combination of nephrotoxic serum plus streptococcal toxin, 9 ml. of streptococcal culture filtrate were added to the total dose of nephrotoxic serum, making 1 ml. of filtrate in each of the 9 doses administered.

During the period of study the animals were housed in separate metabolism cages, and at specified intervals blood and urine samples were obtained. The urine was collected over 24-hour periods. The total proteins in serum and urine were determined by Kingsley's procedure.[7] The electrophoretic pattern of the proteins in the serum and urine was determined by paper strips on the apparatus of Durrum and associates,[8] and quantitation of the paper strips was done by the technique of Grassmann and associates.[9] Several times during the study the Tiselius procedure was used for determination of the electrophoretic patterns of serum and urinary proteins and the patterns were compared with those obtained by the paper technique. The procedure of Zak and associates[10] was used for determination of total serum cholesterol, and that of Van Slyke and Cullen[11] for blood urea.

After establishment of control electrophoretic patterns and values for serum and urinary proteins and the total serum cholesterol and blood urea, the individual animals in each cage were given the nephrotoxic agents as indicated in an earlier paragraph. One hour before receiving the nephrotoxic serum alone or in combination with streptococcal culture filtrate, each of the dogs was given subcutaneously 25 mg. of tripelennamine hydrochloride.*

RESULTS

In this study the effects of the nephrotoxic serum, prepared from rabbits sensitized against dog kidney homogenate, were uniform and the features of the experimental disease were similar to those previously reported by Stickler and us.[1] The dogs herein reported which received the nephrotoxic serum alone in total amounts of 4.4 ml. per kilogram of body weight over a period of 3 days exhibited severe hypoproteinemia with reduction of serum albumin and alpha-1 and gamma globulin, and variable but usually some increase of the alpha-2 and beta globulin. These manifestations appeared on the first day after the first injection and persisted for some time thereafter (Fig. 1). Usually the peak effect appeared within 2 days after the last injection of the nephrotoxic agent. The serum cholesterol and blood urea were consistently elevated soon after the course of injections of nephrotoxic agents was completed.

The various blood proteins appeared in the urine and the proteinuria became most severe immediately after completion of the 3-day course of injections of nephrotoxic serum. Fig. 1 demonstrates the control levels and the comparative effects of the nephrotoxic agents on the serum and urinary proteins, on the serum cholesterol and on the blood urea for more than 40 days. With few exceptions the effects of injecting nephrotoxic serum alone were much like those of the combination of nephrotoxic serum and streptococcal culture filtrate (Fig. 1). The following were the exceptions: The proteinuria induced by the injection of nephrotoxic serum alone completely disappeared by the twentieth day after administration of nephrotoxic serum, while the proteinuria which followed the combination of nephrotoxic serum and streptococcal toxin lasted much longer. In these animals proteinuria persisted

*Pyribenzamine hydrochloride, Ciba Pharmaceutical Products, Inc., Summit, N. J.

throughout the observation period, which lasted for 40 days after the last injection of nephrotoxic serum and streptococcal toxin (Fig. 1). The serum cholesterol and blood urea were slightly higher in the dogs that received the combination than in the dogs that received the nephrotoxic serum alone.

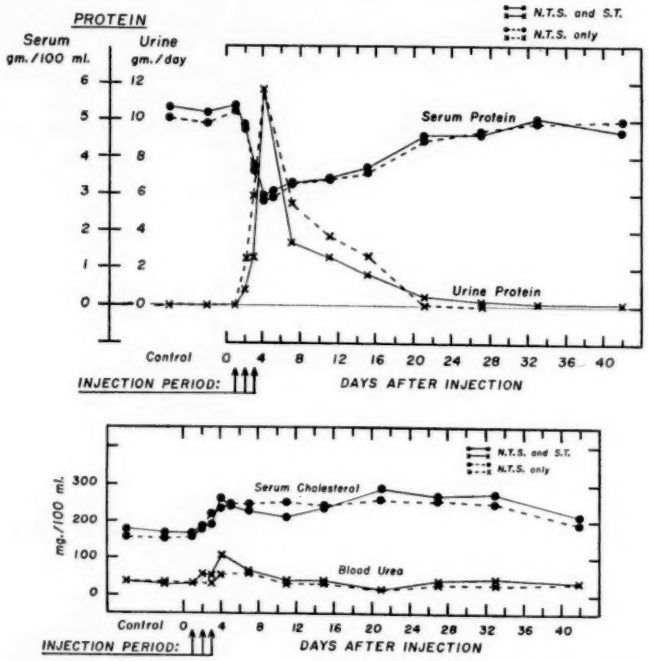

Fig. 1.—Comparative changes in certain constituents of blood and urine following intravenous administration of nephrotoxic serum (N.T.S.) and streptococcal toxin (S.T).

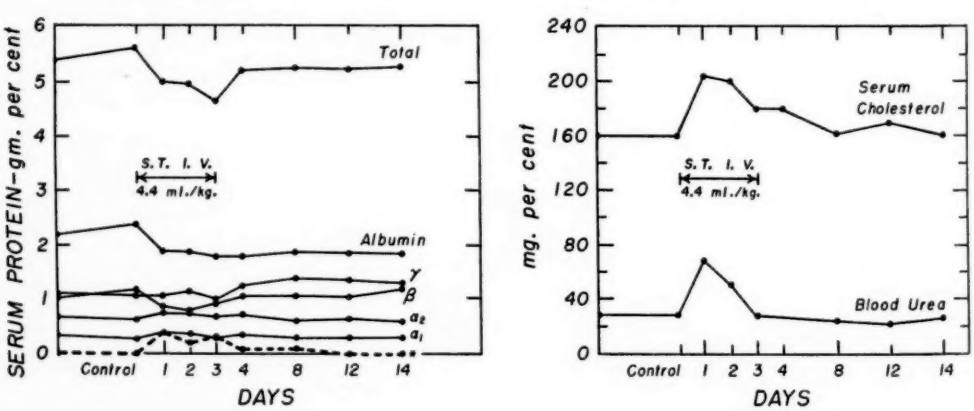

Fig. 2.—Effects on dogs of intravenous administration of streptococcal toxin.

The administration of streptococcal culture filtrate alone, in total amounts of 4.4 ml. per kilogram of body weight given over a 3-day period, produced changes that were similar to those caused by nephrotoxic serum but were

much milder and lasted for a much shorter duration. Fig. 2 demonstrates the various changes in the blood and urine following the intravenous administration of streptococcal culture filtrate alone. During and immediately after the period of administration of streptococcal toxin there occurred a definite but moderate reduction of total serum proteins and of serum albumin (Fig. 2). The serum cholesterol and blood urea were definitely elevated, but for each the increase was transient (Fig. 2). Proteinuria occurred on the first day of injection of streptococcal toxin and lasted until the eighth day only; thereafter, no protein was found in the urine of the dogs which received streptococcal culture filtrate alone (Fig. 2). There was a definite reduction of the serum albumin with a definite increase of the blood urea and serum cholesterol, but the variations in the other fractions of the serum proteins were not consistent. In order to determine the effects of higher concentrations of streptococcal toxin, use of larger doses is planned in a future study.

TABLE I. INFLUENCE OF INTRAVENOUS ADMINISTRATION OF NEPHROTOXIC AGENTS ON CERTAIN CONSTITUENTS OF BLOOD AND URINE

	NEPHROTOXIC SERUM			STREPTOCOCCAL TOXIN			NEPHROTOXIC SERUM AND STREPTOCOCCAL TOXIN		
		MAXIMAL CHANGE			MAXIMAL CHANGE			MAXIMAL CHANGE	
CONSTITUENT	CONTROL*	AMOUNT*	PER CENT	CONTROL*	AMOUNT*	PER CENT	CONTROL*	AMOUNT*	PER CENT
Serum									
Total protein	5.00	−2.2	−44	5.53	−0.87	−16	5.30	−2.34	−44
Albumin	2.51	−1.65	−66	2.31	−0.51	−22	2.69	−1.83	−68
Alpha-1 globulin	0.36	−0.09	−25	0.32	−0.04	−13	0.34	−0.08	−24
Alpha-2 globulin	0.55	+0.09	+16	0.67	+0.09	+13	0.57	+0.30	+53
Beta globulin	1.03	+0.11	+11	1.11	+0.14	+13	1.07	−0.46	−43
Gamma globulin	0.61	−0.19	−31	1.10	−0.14	−13	0.63	−0.28	−44
Cholesterol (mg./100 ml.)	155	+103	+66	161	+43	+27	170	+118	+69
Blood urea (mg./100 ml.)	38	+59	+155	28	+40	+143	33	+75	+227
Urine protein (Gm./24 hr.)	0	11.6		0	0.32		0	11.6	

*Grams per 100 ml., unless otherwise indicated.

In our hands the paper procedure for the determination of the electrophoretic pattern of serum and urinary proteins worked very satisfactorily. In order to test the reliability of the paper procedure for electrophoresis, we applied the two procedures (paper electrophoresis and Tiselius electrophoresis) on the same specimens of serum and urine from the same animals before and after production of experimental nephrosis. Fig. 3 demonstrates the similarity of the findings by the two procedures, giving the various changes in blood and urinary proteins resulting from the nephrosis induced by the nephrotoxic agents used in this study. The curves obtained by the Tiselius and by the paper electrophoretic apparatus were much alike. The repeatability of such results and the close similarity of the findings by the two procedures gave us confidence in the paper procedure and made us conclude that it is reliable to use in the study of electrophoretic patterns of blood and urine.

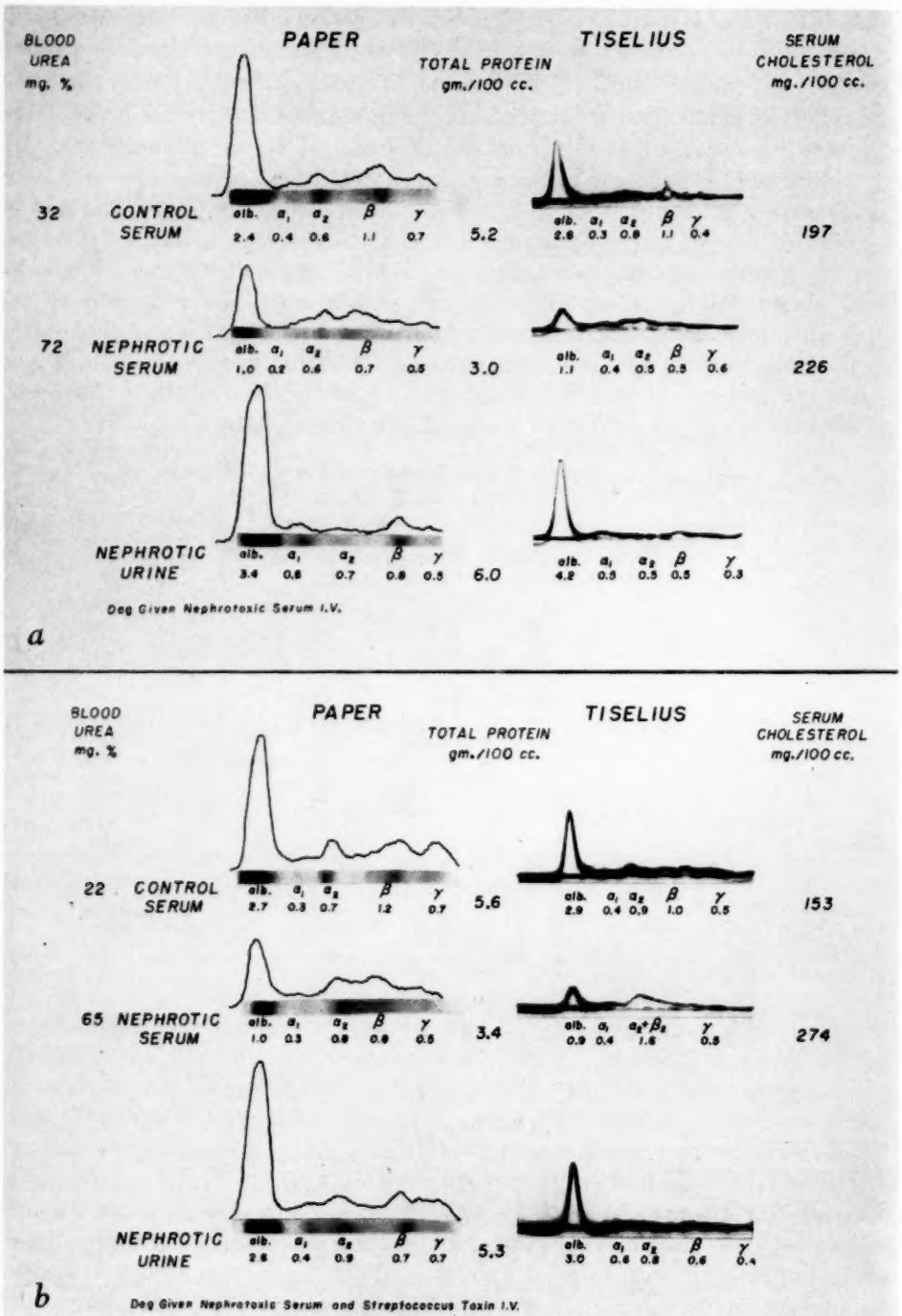
BLOOD UREA mg. %
PAPER
TISELIUS
TOTAL PROTEIN gm./100 cc.
SERUM CHOLESTEROL mg./100 cc.
32 CONTROL SERUM
alb. 2.4 α1 0.4 α2 0.6 β 1.1 γ 0.7
5.2
alb. 2.6 α1 0.3 α2 0.9 β 1.1 γ 0.4
197
72 NEPHROTIC SERUM
alb. 1.0 α1 0.2 α2 0.6 β 0.7 γ 0.5
3.0
alb. 1.1 α1 0.4 α2 0.5 β 0.5 γ 0.6
226
NEPHROTIC URINE
alb. 3.4 α1 0.8 α2 0.7 β 0.8 γ 0.5
6.0
alb. 4.2 α1 0.5 α2 0.5 β 0.5 γ 0.3
Dog Given Nephrotoxic Serum I.V.
a
BLOOD UREA mg. %
PAPER
TISELIUS
TOTAL PROTEIN gm./100 cc.
SERUM CHOLESTEROL mg./100 cc.
22 CONTROL SERUM
alb. 2.7 α1 0.3 α2 0.7 β 1.2 γ 0.7
5.6
alb. 2.9 α1 0.4 α2 0.9 β 1.0 γ 0.5
153
65 NEPHROTIC SERUM
alb. 1.0 α1 0.3 α2 0.8 β 0.8 γ 0.5
3.4
alb. 0.9 α1 0.4 α2+β2 1.6 γ 0.5
274
NEPHROTIC URINE
alb. 2.6 α1 0.4 α2 0.9 β 0.7 γ 0.7
5.3
alb. 3.0 α1 0.6 α2 0.8 β 0.6 γ 0.4
Dog Given Nephrotoxic Serum and Streptococcus Toxin I.V.
b

Fig. 3.—Electrophoretic patterns of the same samples of serum and urine determined by two different procedures (paper electrophoresis and Tiselius electrophoresis). *a*, Samples from dog given nephrotoxic serum intravenously. *b*, Samples from dog given nephrotoxic serum and streptococcal toxin intravenously.

Table I demonstrates the maximal changes produced by the nephrotoxic agents used in this study. Not shown in the table is the fact that these maximal changes usually occurred on the first or second day after completion of the 3-day course of injections of the nephrotoxic agents. The table gives in percentage the maximal changes from the control values that had been obtained before administration of the nephrotoxic agents. Note that the combination of nephrotoxic serum and streptococcal toxin produced the greatest increase of blood urea (227 per cent) and of alpha-2 globulin (53 per cent), and also the greatest decrease of gamma globulin (–44 per cent). The association of gamma globulin with immunity reactions makes this reduction of gamma globulin in the serum a basic explanation for the susceptibility to infection encountered in such nephrotic states.

SUMMARY

On healthy immunized young dogs a study was made of the influence of streptococcal toxin on the course of experimental nephrosis induced by the intravenous administration of serum obtained from rabbits sensitized to dog kidney. After establishment of the control electrophoretic patterns and control values for serum proteins, serum cholesterol, blood urea, and urinary proteins, the dogs were divided into 3 equal groups: Group I was given rabbit nephrotoxic serum; Group II was given streptococcal culture filtrate; and Group III was given a mixture of rabbit nephrotoxic serum and streptococcal culture filtrate. The total dose of each of the nephrotoxic serum and of the streptococcal culture filtrate was 4.4 ml. per kilogram of animal body weight and was divided into 9 doses given intravenously over a period of 3 days. Blood and urine samples were analyzed daily during the first week and 2 or 3 times a week thereafter for more than 40 days. Nephrotoxic serum alone produced a uniform picture characterized by severe hypoproteinemia, with reduction of serum albumin, alpha-1 and gamma globulins, and variable but usually some increase in the alpha-2 and beta globulins. The serum cholesterol and blood urea were consistently elevated. Proteinuria was severe but disappeared by the twentieth day after administration of nephrotoxic serum. These manifestations were most marked immediately on completion of the course of injections of nephrotoxic serum.

The intravenous administration of streptococcal culture filtrate alone produced changes which were similar to those caused by nephrotoxic serum but were very much milder and lasted for a much shorter duration. However, intravenous administration of the combination of nephrotoxic serum and streptococcal toxin produced a picture much like that of nephrotoxic serum alone with the following exceptions: the proteinuria which followed the use of the combination of nephrotoxic serum and streptococcal toxin persisted much longer (more than 40 days), and the reduction of gamma globulin was greater and the increase of serum cholesterol and blood urea was slightly higher than in the dogs which received nephrotoxic serum only.

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THE PLASMA AMINO ACIDS IN PATIENTS WITH LIVER FAILURE

FRANK L. IBER, M.D., HYMAN ROSEN, M.A., STANLEY M. LEVENSON, M.D., AND THOMAS C. CHALMERS, M.D.
WASHINGTON, D. C.

IN 1849 Rokitansky[1] recognized crystals of tyrosine and leucine in the liver of patients with acute hepatic necrosis, but the importance of the liver in amino acid metabolism was not clearly revealed until 1924, when Bollman, Mann, and Magath[2] showed that the hepatectomized dog was unable to form urea from amino acids. Although the total plasma amino acids are normal in mild liver disease, elevations are usually found in severe necrotizing hepatic disease.[3]

The quantitative partition of the blood amino acids into individual components by column chromatography has only recently become possible.[4, 5] Reports of the behavior of individual plasma amino acids in patients with liver disease[6-8] or in experimental liver disease[9, 10] are limited. This paper presents individual plasma amino acid levels in 2 normal subjects and in 7 patients with liver disease of varying severity; 5 of these patients were in liver coma. The effects of intravenous sodium glutamate are noted in the 2 normal subjects and in 3 of the patients in hepatic coma.

MATERIALS AND METHODS

Venous blood samples were collected in citrate or heparin. The plasma was immediately separated by centrifugation, frozen, and stored at −18° C. until used. The plasma was deproteinized by ultrafiltration through "Visking" cellophane under nitrogen pressure in a refrigerated Monel metal apparatus.[11] This apparatus separates approximately 70 per cent of the initial plasma volume as filtrate. Chromatography was then performed on Dowex 50 columns according to the technique of Moore and Stein.[4, 5] The amino acids were determined on the eluate by the colorimetric method of Stein and Moore[12] using a standard correction for each amino acid. Glutamine is lost in varying degrees on the column, and glutamine, asparagine, and serine (G.A.S. complex) come off together. Glutamine was determined separately by enzyme hydrolysis with glutaminase derived from a rat kidney homogenate.[13] The normal values by this method range from 350 to 760 μM/L. Alpha-ketoglutarate, pyruvate, and ammonia were determined by the methods of Seligson.[14] Citric acid was determined by the method of Ettinger, Goldbaum, and Smith.[15]

Pertinent information on patients used in this study is given in Table I. Patients 1, 2, 5, 6, and 7 received an intravenous infusion of monosodium glutamate.* All of these patients received a control infusion of glucose before and after sodium glutamate administration. The glutamate was given in 5 per cent glucose at such a rate that the amount of glucose per unit time during this and the control periods remained constant. Patients 1, 2, 5, 6, and 8 represent Patients 1, 2, 4, 5, and 10, respectively, in another paper.[16]

From The Department of Metabolism, Division of Medicine, Department of Surgical Metabolism, Division of Surgery, Walter Reed Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.

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*The monosodium glutamate was generously provided by the Sherman Laboratories, Detroit, Mich.

TABLE I. PERTINENT CLINICAL INFORMATION ON THE NORMAL SUBJECTS AND PATIENTS WITH CIRRHOSIS

NO.	AGE	SEX	DIAGNOSIS	DURATION (MONTHS)	ALBUMIN (GM./100 ML.)	BODY NUTRITION AND FOOD INTAKE	BILIRUBIN (MG./100 ML.)	MENTAL STATE	FACTOR PRECIPITATING COMA	OUTCOME
1	21	M	Normal	—	5.0	Good	0.5	Clear	—	—
2	26	M	Normal	—	4.6	Good	0.3	Clear	—	—
3	51	M	Mild cirrhosis; toxic hepatitis	0.5	2.6	Good Mild anorexia	7.0	Precoma	p-amino salicylic acid	Recovered
4	17	F	Wilson's disease; cirrhosis (severe)	12	1.4	Poor No intake, 6 days	14.0	Precoma Coma, 24 hr.	(?) Paracentesis	Died, 3 days
5	62	M	Cirrhosis (severe)	18	3.4	Poor No intake, 18 days	31.8	Coma, 24 hr.	Hemorrhage	Died, 3 days
6	44	M	Cirrhosis (severe)	0.5	2.8	Poor No intake, 10 days	19.0	Coma, 12 hr.	(?) Paraldehyde	Died, 12 days
7	46	M	Cirrhosis (severe)	4	2.2	Poor Mild anorexia	3.2	Coma, 6 hr.	Hemorrhage	Died, 1 day
8	52	M	Nonalcoholic cirrhosis (severe)	8	1.4	Poor No intake, 6 days	4.4	Coma, 24 hr.	(?) I.V. protein hydrolysate	Died, 15 days
9	12	M	Wilson's disease; cirrhosis (mild)	12	3.4	Good	0.6	Clear	—	Alive

RESULTS

All data are given in Table II. Determination 1-A and 2-A were performed in normal subjects who were receiving infusions of glucose. The values for each of the individual amino acids were within or below the range found in 17 normal subjects in whom plasma was obtained after a 6-hour fast. Determination 9-A was in a patient with well-compensated cirrhosis; the plasma amino acid pattern was almost normal—proline, glycine, histidine, and leucine concentrations were moderately depressed and tyrosine was increased twofold. Determinations 3-A and B, and 4-A were in patients with severe liver disease in precoma. In 3-A and B, although the total amino acid level seemed normal, taurine was elevated about twofold, and threonine, G.A.S. complex, glycine, methionine, and histidine were mildly depressed. In 4-A the total amino acid was definitely elevated but there were particular elevations in aspartic acid, threonine, methionine, tyrosine, phenylalanine, and lysine. Patient 4, who was treated symptomatically, progressed into hepatic coma (at the time of 4-B) and died. In determination 4-B, 36 hours after 4-A, the total amino acids increased threefold, but aspartic acid and glutamic acid fell. Alanine, proline, cystine, and histidine showed the most marked rises (four- to eightfold); taurine, G.A.S. complex, glycine, methionine, tyrosine, phenylalanine, and lysine rose to lesser degrees.

Determination 6-A was performed on a patient in light hepatic coma with underlying cirrhosis. The total plasma amino acid level was markedly decreased; glutamic acid, proline, valine, methionine, isoleucine, tyrosine, phenylalanine, and histidine were particularly depressed.

Determinations 4-B, 5-A, 7-A, and 8-A were performed in different patients in severe hepatic coma. All 4 subjects showed increased levels of methionine (two to twentyfold) and tyrosine (two- to fivefold). Three of the 4 subjects showed increased levels of phenylalanine (two- to fivefold) and threonine (one and one-half to sevenfold).

Two normal subjects and 3 patients with hepatic coma had plasma amino acids measured before and after an infusion of glutamate. In the 2 normal subjects, the glutamic acid level rose to 1,700 and 1,130 μM/L., respectively, near the end of the infusion. The glutamic acid level fell rapidly after completion of the infusion and, in Patient 1, returned to normal within two hours. The level of glutamate fell to one-third of the maximal value within 15 minutes in Patient 2. Both normal subjects showed a rise in alanine and in G.A.S. complex, the latter persisting longer than the former. Aspartic acid rose in the one subject in whom it was measured. There were mild rises in other amino acids following glutamate infusion.

In the comatose subjects (Patients 5, 6, and 8) the second determination was made immediately after the infusion of the glutamate, but only in Subject 6 did the glutamic acid level exceed that observed in the 2 normal subjects. All 3 of the coma patients showed a rise in alanine (50 to 350 per cent of the control value). Two of the 3 patients showed a rise in G.A.S. complex (40 and

TABLE

	NORMALS* ± S.D.	PATIENT 1† NORMAL A	PATIENT 1† NORMAL B	PATIENT 1† NORMAL C	PATIENT 2‡ NORMAL A	PATIENT 2‡ NORMAL B	PATIENT 2‡ NORMAL C	PATIENT 2‡ NORMAL D
μM of Amino acid N/L. plasma ultrafiltrate								
Taurine	48 ± 10	28	55	43				
Aspartic acid	24 ± 10	12	61	23				
Threonine	131 ± 20	79	91	80	79	78	73	76
Glutamine, asparagine, serine	498 ± 60	454	520	540	168	210	215	87
Glutamic acid	138 ± 80	91	1700	85	112	1130	775	400
Proline	230 ± 40	105	118	103				
Glycine	241 ± 20	153	155	147	82	100	89	57
Alanine	319 ± 40	285	408	282	200	305	235	182
Valine	230 ± 50	172	173	186				
Methionine	22 ± 5	25	20	23				
Isoleucine	75 ± 5	56	55	49				
Leucine	116 ± 5	107	117	106				
Tyrosine	60 ± 5	34	41	32				
Phenylalanine	71 ± 5	31	43	31				
Cystine	Trace	Trace	Trace	Trace				
Histidine	141 ± 20	121	46	72				
Lysine	161 ± 20	152	134	88				
Total**	2505 ± 400	1905	3737	1890				
μM/L. whole blood								
Ammonia (NH_3)	67.6 ± 10	166	72	52				
α-Ketoglutarate	13.0 ± 1.5	12.4	20.6	13.7	11.3	17.8	16.0	11.8
Pyruvate	73 ± 16	115	128	90	69.1	71.0	67.5	78.8
Citrate	79.2	70	58	82	80	110	127	109
Glucose mg./100 ml.		88	96	95	77	82	83	80
Glutamine		229	265					

*Amino acid normals were determined on 17 individuals. The ammonia normals were determined in 25 individuals. The pyruvate and alpha-ketoglutarate are based on 16 persons. See methods for citrate and glutamine.

†I-A, sample drawn at the end of glucose infusion and just prior to the glutamate infusion. 1-B drawn at end of glutamate infusion (24 Gm. in 195 min.). 1-C drawn 120 min. after 1-B.

‡2-A drawn at end of glucose infusion and just before glutamate infusion. 2-B after 200 min. of glutamate infusion (24 Gm. in 205 min.). Glutamate infusion continued for 5 min. after 2-B. 2-C drawn 5 min. after glutamate infusion ceased. 2-D drawn 15 min. after end of glutamate infusion.

§Specimen obtained 7 days after A.

200 per cent) and in aspartic acid (40 and 500 per cent). Two of the three had a rise in the total amino acid level of 2 and 8 times the control; the third subject had no significant change.

Citric acid, pyruvic acid, and α-ketoglutaric acid were elevated in the cases with severe liver failure. α-Ketoglutaric acid was the only one of the three that rose consistently following glutamate infusion.

DISCUSSION

Fig. 1 shows in a schematic fashion the principal tissues which affect the plasma level of amino acids. The liver plays a central role in amino acid metabolism, for it can release amino acids into the plasma; convert some amino acids to others; control, in part, the release of dietary amino acids into the peripheral blood stream; and remove amino acids from the plasma. Muscle and other tissues may also release amino acids into the blood stream or take them from the blood stream for the building of new protein or for energy. The

II

PATIENT 3 PRECOMA		PATIENT 4‖ COMA		PATIENT 5¶ COMA		PATIENT 6¶ COMA		PATIENT 7 COMA	PATIENT 8¶ COMA		PATIENT 9 CIRRHOSIS
A	B§	A	B	A	B	A	B	A	A	B	A
78	79	33	100	55	47	70	135	34	50	32	50
15	Trace	192	37	41	60	23	124	23	22	17	12
65	66	550	1083	182	226	53	111	204	147	151	116
295	230	920	2550	470	1030	144	85	1038	420	600	267
61	60	221	175	152	957	17	3730	129	157	191	140
208	111	240	1080	233	176	20	8	250	153	155	103
131	151	510	1410	366	387	87	155	300	286	262	132
243	220	430	3260	485	1490	75	280	410	230	324	297
138	67	150	373	130	182	20	49	166	137	98	146
13	Trace	225	534	41	52	Trace	Trace	105	45	47	37
46	76	66	168	60	106	6	Trace	50	27	32	37
75	119	110	313	82	173	27	42	106	53	71	67
77	61	130	349	126	262	Trace	8	175	169	138	173
87	43	124	438	185	148	3	12	188	90	60	67
Trace	Trace	Trace	350	Trace	Trace	Trace	Trace	5	Trace	Trace	12
42	101	182	830	229	170	5	5	308	60	92	32
147	95	455	1310	Not done		13	17	248	210	138	101
1721	1479	4538	14360	2837	5466	563	4761	3739	2256	2408	1789
60		57	172	140	88	148	102	320	191	158	82
39.8		57	43	30	144	53	76	37	24	19	13
148		175	620	156	260	222	106	355	122	108	75
235		230	276	73	122	128	114	368			117
88		114	177	93	122	91	92	53	208	178	83
				700	1240				1350	1440	

‖4-A precoma present for 12 hr. 4-B 36 hr. after 4-A. Patient in deep coma for 24 hr.

¶5-A, 6-A, and 8-A obtained before glutamate infusion was started and at the end of the glucose infusion. 5-B at the end of glutamate infusion (24 Gm. in 2½ hrs.). 6-B after 32 Gm. in 4½ hr. 8-B after 20 Gm. in 3½ hr.

**Total obtained from addition of the amino acids listed.

kidney, in addition, transforms certain amino acids into others (such as glutamine to glutamic acid) and can excrete amino acids. At present not all of the factors that lead to the excretion of amino acids by the kidney are known.[10, 17]

In Fig. 1, amino acids from absorption are shown as passing through the liver before reaching the plasma amino acid pool. This is not entirely correct because shunting of the blood around the liver often occurs in liver disease. Recent observations[18] indicate that significant amino acid absorption occurs by way of the thoracic duct; thus, in effect, a portion of the amino acids of absorption may be "shunted" to the systemic circulation in the normal.

In the data recorded in this paper, the rise in methionine and the rise in tyrosine were the most persistent of all of the individual changes of the plasma amino acids. Wu, Bollman, and Butt[7] found both of these amino acids elevated and, in addition, found glutamic acid, glutamine, and cystine high in liver coma. Walshe[6] reported a rise in methionine and glutamine and emphasized the diagnostic value of an elevated tyrosine in the urine in severe liver disease. Cachin, Durlach, and Blass[8] found that elevated tyrosine was the earliest change in the plasma in liver disease, and Schreier[19] emphasized the rise in

plasma methionine in liver failure. The rise in both methionine and tyrosine in our patients is out of proportion to the rise in the total plasma amino acids. Methionine was depressed in several of the patients with milder liver failure, confirming the observation of Wu, Bollman, and Butt.[7]

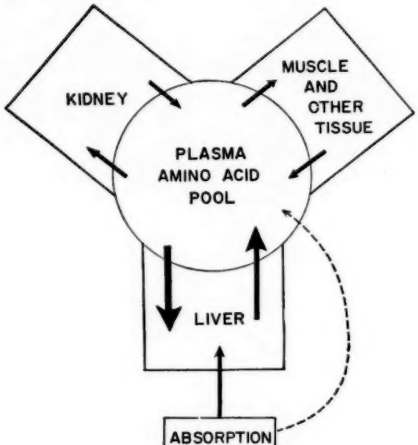

Fig. 1.—Schematic representation of the principal tissues that affect the plasma amino acid pool.

Four sources of the rise in plasma amino acids after severe liver damage seem possible: (1) dissolution of necrotic liver tissue,[20] (2) release from muscle or other tissue protein, (3) release from free amino acid pools in the tissues,[10] or (4) impaired utilization of plasma amino acids. Plasma amino acids rise at least as rapidly following hepatectomy as following severe liver necrosis[21] and both increases may be lessened by giving glucose.[10] The amino acid distribution of whole liver protein does not parallel the distribution of the rise in the plasma amino acids.[6] Dissolution of necrotic liver tissue does not, therefore, seem the source of the elevated amino acids. Flock, Mann, and Bollman[21] found that the rise in amino acids following hepatectomy occurred in the same proportion as in normal plasma, and they favored the theory that release of amino acids occurred from the free amino acid pool of muscle. In a later study,[22] however, they found that muscle and brain had a net increase in free amino acids; therefore some release from body protein had occurred. Of the possibilities, increased release of amino acids from muscle protein and impaired utilization by the liver are the most probable.

Many pathways of amino acid utilization occur in the liver, but of these information is available only on urea formation. Although urea formation has been found deficient in rare instances of liver failure,[23] it is usually normal.[24] Blood urea values were determined in Patients 4, 5, 7, 8, and 9 and were either normal or elevated. Altered energy production has often been observed in the presence of severe liver disease[25] and an elevation of the plasma amino acid

level might be expected to correct this deficiency in part. The known actions of cortisone on protein metabolism[26, 27] would make the adrenal steroids possible mediators of this release of amino acids.

In animal studies, the increment above normal in plasma amino acids is similar in composition to the normal plasma amino acids and to amino acids of muscle tissue.[21] In the human being, glutamic acid, alanine, glycine, lysine, aspartic acid, proline, valine, isoleucine, and leucine are found in high molar concentration in whole muscle.[28] In liver failure, none of these show a rise in plasma out of proportion to the rise of the total plasma amino acids; lysine is a possible exception. If muscle protein were the source of the additional plasma amino acids, then there must be alterations in the subsequent metabolism of individual amino acids to account for the lack of rise or the excessive rise of a given amino acid.

The patients with mild liver failure did not show consistent abnormalities. The total amino acids seemed normal in all except Case 6, in which an extremely low value was found. This is unexplained but may be a manifestation of poor nutrition, for the subject had no oral intake for 10 days. Charkey, Kano, and Houghan[29] found a persistent fall in most of the individual plasma amino acid levels in normal subjects fasted for 48 hours. Patient 6 had inadequate calories for maintenance of body weight and no nitrogen intake for 10 days prior to study. However, Patient 5 underwent a comparable fast without depression of the total amino acids. Mellinkoff, Boyle, and Frankland[30] found a more rapid disappearance of infused amino acids in mild liver disease than in normal persons. They suggested that decreased ability to mobilize or store glucose led to a higher output of insulin, which in turn accelerated the fall in amino acids. We have no data to support this concept.

Subjects 1 and 2 received sodium glutamate infusions, and at the end of the infusion the levels were 1,700 and 1,300 μM/L., respectively. The dose used in this study (24 Gm.) would represent 2,300 μM/L. in 55 liters, the approximate total body water of Patient 2, a man weighing 82 Kg. The plasma levels of this amino acid returned rapidly to normal after the end of the infusion. Associated with the fall in glutamic acid, the G.A.S. complex and alanine rose in both patients, and aspartic acid and leucine rose in the single patient in which they were measured. A rise in alanine out of proportion to the rise in other amino acids was found in all three of the coma patients treated with glutamic acid. Alanine most likely is formed as a result of the transamination reaction, in which one molecule each of glutamic acid and pyruvic acid combine to form one molecule each of alanine and α-ketoglutarate. α-Ketoglutarate rose after glutamate infusion in each patient. The rise in alanine and ketoglutarate following glutamate infusion was similar in normals and patients in liver coma.

There are several differences in the metabolism of amino acids in the patients with liver coma and the normal subjects. The total plasma amino acids are occasionally elevated in severe liver failure. Two of the five patients in hepatic coma showed significant elevations. The plasma concentration of methionine is disproportionately elevated in severe liver disease. A defect in

the oxidation of methionine to cystine may be the difficulty, for serine, cystine, and taurine did not show consistent changes. Wu, Bollman, and Butt,[7] in contrast, found an elevation of cystine. The tyrosine concentration is elevated in severe liver failure. Most of the cases showed, in addition, an accumulation of phenylalanine, indicating that oxidation of both substances may be defective.

In contrast to the limited number of differences in amino acid metabolism, the patient with a failing liver and the normal person have a great number of similarities. An infused load of glutamate produced comparable elevations in the glutamic acid levels in both the normal subjects and the patients in coma. Alanine levels rose in both, and the other amino acids changed in no persistent pattern.

SUMMARY

Individual plasma amino acids were studied by column chromatography in 7 individuals with liver disease in various states of decompensation.

1. The plasma amino acids rose with increasing severity of the liver disease, but were not always elevated in hepatic coma.

2. Methionine and tyrosine were disproportionally elevated, and this was the most common defect in the individual amino acids found in all of the 4 patients with liver coma. Phenylalanine accumulation was found in 3 of 4 patients in liver failure.

3. Sodium glutamate was metabolized rapidly when given intravenously and apparently was metabolized in the same manner in normal subjects and patients with severe liver failure.

4. Following infusion of glutamate there was a rise in all plasma amino acids, but alanine and glutamic acid showed the greatest increases.

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EFFECT ON PORTAL BLOOD AMMONIUM CONCENTRATION OF ADMINISTERING METHIONINE TO PATIENTS WITH HEPATIC CIRRHOSIS

LESLIE T. WEBSTER, JR., M.D.,* AND GEORGE J. GABUZDA, M.D.
CLEVELAND, OHIO

IN CERTAIN patients with hepatic cirrhosis or Eck fistula, the administration of ammonium salts, protein, amino acids, or urea may induce impending hepatic coma, a syndrome characterized by mental confusion and a typical flapping tremor and usually associated with increased blood ammonium concentrations.[1-6] These nitrogenous substances may produce increased blood ammonium concentrations in similar patients without inducing the above untoward clinical signs.[5, 7, 8] Confusion also may follow methionine ingestion by patients with hepatic disease.[9-11] However, metabolites of methionine other than ammonium have been implicated by some investigators because blood ammonium concentrations did not change significantly in methionine-fed patients with cirrhosis, although some exhibited neurological deterioration. These metabolites were presumably formed by gastrointestinal bacterial action on methionine because methionine-induced neurological deterioration was prevented by chlortetracycline administration.[12]

In the present study, methionine as a source of portal blood ammonium was evaluated by determining arterial and portal collateral venous blood ammonium concentrations before and after methionine ingestion by patients with cirrhosis. Increases in portal collateral blood ammonium concentrations were observed after ingestion of this amino acid. Consistently significant increases were not observed after methionine ingestion when the same patients were pretreated with neomycin sulfate by the oral route.

MATERIALS AND METHODS

Three hospitalized male patients with histories of chronic alcoholism associated with poor food intake were studied. Clinical, laboratory, and histologic evidence indicated diagnoses of Laennec's cirrhosis. No patient had confusion, coma, tremor, shock, hemorrhage, or blood urea nitrogen concentrations above 15 mg. per 100 ml. (Table I).

The daily food of these patients was estimated to contain 30 to 90 Gm. of protein and approximately 250 mg. of sodium; there were no substantial dietary alterations during the observation period. Patients did not receive foods or medications containing nitrogen for at least 12 hours before each study was initiated.

Each study was performed by obtaining blood samples for ammonium-nitrogen determinations within two minutes of each other from a superficial abdominal vein and a brachial

From the Departments of Medicine, Western Reserve University School of Medicine, and the City Hospital, Cleveland, Ohio.

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*Russell M. Wilder Fellow of the National Vitamin Foundation.

artery at intervals before and after the ingestion of 10 Gm. of DL-methionine* in 250 ml. of water. Two studies were carried out for each patient. On the afternoon of the day following their first study, they were given neomycin sulfate† orally, 2 Gm. initially, followed by 1 or 2 Gm. every 4 to 6 hours, until the next morning when the second study was done.

Ammonium-nitrogen (NH_4-N)‡ in blood and in the administered methionine solution was determined by a modified Conway technique.[6, 13] Blood was injected promptly into the Conway unit and a diffusion time of 10 minutes was employed. Results were calculated from the NH_4-N recovered from a standard as recommended for blood by Conway. Differences between NH_4-N concentrations in two consecutive blood samples drawn during the same day from the same site in a given fasting patient with cirrhosis ranged from 0 to 23 meg. per 100 ml. This reproducibility held for superficial abdominal venous blood only if the blood was drawn repeatedly from the same location in the same abdominal vein. Accordingly, multiple sampling of abdominal venous blood was done in this manner.[8]

The 10 Gm. of methionine yielded 0.1 mg. of NH_4-N on analysis.

Serum bilirubin and blood urea nitrogen concentrations were determined by standard techniques.[14, 15] Bromsulphalein was determined in serum from blood obtained 45 minutes after the injection of 5 mg. of dye per kilogram of body weight.[16]

TABLE I. CLINICAL AND LABORATORY DATA IN THREE MALE PATIENTS WITH HEPATIC CIRRHOSIS*

PATIENT AGE	PHYSICAL EXAMINATION†	SERUM TOTAL BILIRUBIN (MG./100 ML.)	SERUM BSP (PER CENT RETENTION)	BLOOD UREA-N (MG./100 ML.)
A. I. 58	Angiomata, hepatomegaly, splenomegaly	2.0	38	13
J. K. 49	Icterus, angiomata, hepatomegaly, edema	3.0	53	12
E. R. 55	Edema	1.8	38	12

*Diagnoses of Laennec's cirrhosis were confirmed by biopsy.
†All three patients had ascites and dilated superficial abdominal veins.

RESULTS

Before neomycin sulfate administration, the three patients studied demonstrated significant increases in portal collateral blood NH_4-N concentrations ($>$ 23 meg. NH_4-N per 100 ml.) within an hour after methionine ingestion (Figs. 1 to 3). However, after 6 to 12 Gm. of neomycin sulfate had been given over a period of 16 to 18 hours, methionine did not produce significant increases in portal collateral blood NH_4-N concentrations in two patients and produced only a minimal increase in the third. Except in one instance (Fig. 3) arterial blood NH_4-N concentrations did not increase significantly after methionine was ingested either before or after neomycin sulfate administration. Fasting portal collateral and arterial blood NH_4-N concentrations were lower after neomycin sulfate administration in 2 of the 3 patients (Figs. 1 and 3). The significance of this finding was difficult to evaluate because day-to-day variations in a given fasting patient with cirrhosis can be of this magnitude.

*Kindly provided by Merck & Company, Rahway, N. J.
†Mycifradin sulfate, The Upjohn Company, Kalamazoo, Mich.
‡Whether ammonium normally is present in circulating blood or whether it is formed only after blood is shed has not been established. Volatile base, released from blood upon addition of alkali, is assumed to be ammonia.

The superficial abdominal veins employed contained portal blood to a measurable extent as evidenced by the following: Ammonium-nitrogen concentrations in blood obtained from these veins increased significantly and were in excess of increases in arterial blood NH_4-N concentrations in two of the neomycin-treated patients when NH_4Cl was given orally (50 mg. of NH_4-N to J. K. and 100 mg. to E. R.). Similar results were noted when 4 Gm. of L-glutamine were given orally to the third patient (A. I.) who had also received neomycin sulfate. Previous studies have demonstrated that glutamine can cause increased portal collateral blood NH_4-N concentrations in patients treated with neomycin.[8]

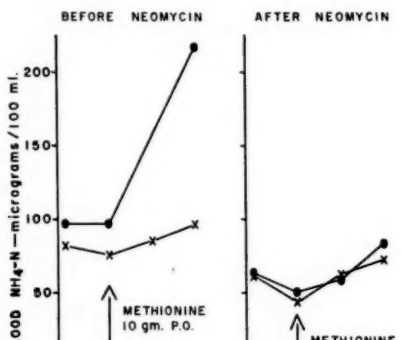

Fig. 1.—Effect of DL-methionine given orally. Patient J. K. with cirrhosis.

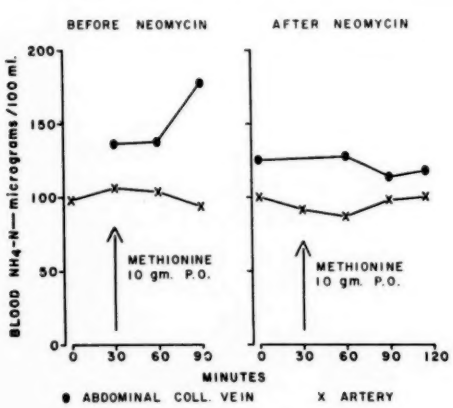

Fig. 2.

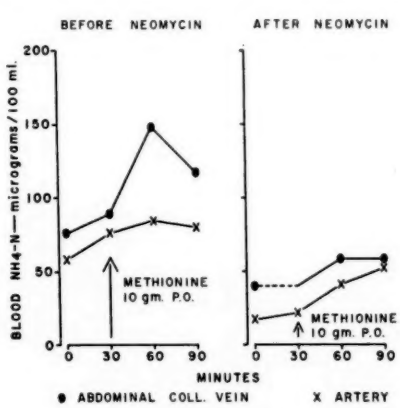

Fig. 3.

Fig. 2.—Effect of DL-methionine given orally. Patient E. R. with cirrhosis.
Fig. 3.—Effect of DL-methionine given orally. Patient A. I. with cirrhosis.

DISCUSSION

Increased concentrations of volatile base (NH_4-N) in portal collateral blood were noted shortly after each of three patients with hepatic cirrhosis ingested 10 Gm. of DL-methionine.

These increases in portal blood NH_4-N were probably not attributable to the NH_4-N found by analysis in the 10 Gm. quantity of methionine administered (0.1 mg.), because 20 mg. of NH_4-N given orally in the form of an ammonium salt did not produce a portal collateral increase in other patients with cirrhosis.[8] Further evidence for this hypothesis is that insignificant increases in portal collateral NH_4-N concentrations followed methionine administration to neomycin-treated patients. Increases from free NH_4-N that might have been contained in methionine would not have been modified by neomycin.[8] This suggests that NH_4-N in detectable quantities was released only after methionine was metabolized.

The fact that no significant increases in portal collateral blood NH_4-N concentrations followed oral administration of neomycin suggests that formation of the NH_4-N from methionine depended, in part at least, upon gastrointestinal bacterial action. For example, methionine might be deaminated by gastrointestinal bacteria or it could be absorbed and changed in part to urea which, upon entering the gastrointestinal tract, might be deaminated by bacterial urease. In either situation, increased portal collateral blood NH_4-N concentrations would result if the bacterial flora were not grossly altered.

The increased portal collateral blood NH_4-N concentrations found here were at variance with other reported results, indicating that blood NH_4-N concentrations were not influenced by ingested methionine.[12] However, the preparations of DL-methionine employed, the dosages of amino acid given, the sites from which blood was taken, and the time intervals of blood sampling were not comparable.

Whether or not the ability of methionine to induce impending hepatic coma is related to its ability to induce increased portal collateral blood NH_4-N concentrations is not known. Other investigators have reported that chlortetracycline may prevent the exacerbation of impending hepatic coma in methionine-fed patients with cirrhosis, presumably by its bacteriostatic effect.[12] In this study, neomycin sulfate, an essentially nonabsorbable antibiotic, prevented increased portal collateral blood NH_4-N concentrations after methionine ingestion, apparently by altering the gastrointestinal flora concerned with ammonium production. These observations are consistent with the hypothesis that ammonium or a related substance may play a role in methionine-induced hepatic coma. However, the possibility of a deleterious effect resulting from other bacteriologically produced metabolites of methionine cannot be eliminated.

Speculations upon the quantitative aspects of gastrointestinal "ammonium" production from methionine contained in protein appear relevant. After ingestion, 20 to 50 Gm. of animal protein, containing an estimated 0.7 to 1.8 Gm. of methionine,* produced increased arterial as well as portal collateral blood NH_4-N concentrations in some patients with cirrhosis.[8] However, in the present study, 10 Gm. of free methionine or about 6 times as much as contained in the above protein meals did not produce a significant increase in arterial blood NH_4-N concentrations except in one instance. Moreover, portal blood NH_4-N

*Approximately 3.5 per cent of animal protein is methionine.[17]

increases after ingested protein, unlike those after methionine, were not modified significantly by prior administration of neomycin.[8] Thus, it appeared unlikely that the quantity of methionine contained in these protein meals accounted for much of the portal blood NH_4-N increase observed after their ingestion. However, in patients with severe liver disease, methionine given repeatedly in quantities sometimes employed therapeutically or as dietary supplements might not only produce increased blood "ammonium" concentrations but also induce the syndrome of impending hepatic coma.

SUMMARY

Ten grams of ingested DL-methionine caused increased concentrations of ammonium or a related substance in the portal blood of each of three fasting patients with hepatic cirrhosis. These increases were largely prevented when each patient received 6 to 12 Gm. of neomycin sulfate orally during a period of 16 to 18 hours before methionine ingestion. These observations indicate that "ammonium" is formed from methionine or one of its metabolites and is absorbed into the portal blood. Furthermore, they suggest that the formation of "ammonium" from methionine is dependent to some extent upon the integrity of the gastrointestinal flora.

Methionine possibly may act like other nitrogenous substances containing or liberating "ammonium" which, like this amino acid, have induced the syndrome of impending hepatic coma in susceptible patients with liver disease or Eck fistula.

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LEUKOCYTE PHOSPHATASE ACTIVITY IN PREGNANCY

JACK A. PRITCHARD, M.D.
DALLAS, TEXAS

THE alkaline phosphatase activity of circulating leukocytes may vary considerably. Marked increases in enzyme activity have been observed in several pathologic states including infection, trauma, hemorrhage, and pulmonary and myocardial infarctions, while in myeloid leukemia the enzyme activity is depressed.[1-3] The administration of corticotropin or cortisone to normal individuals results in considerable augmentation of leukocyte alkaline phosphatase activity.[2] The acid phosphatase activity of leukocytes, however, does not appear to vary appreciably from normal in these several conditions.[1, 2]

This report concerns the effects of normal pregnancy with its associated hormonal and hematological alterations on leukocyte alkaline and acid phosphatase activity.

MATERIAL AND METHODS

Leukocytes were obtained and their alkaline and acid phosphatase activities measured following the procedures of Valentine and Beck.[1] It was not necessary to add fibrinogen solution to blood from most women in the latter half of pregnancy since the sedimentation rate was quite rapid, undoubtedly due to the hyperfibrinogenemia of normal pregnancy. White blood cell counts were done in duplicate.

Leukocyte alkaline and acid phosphatase activities and white blood cell counts were determined in 12 healthy nonpregnant women, and in 81 apparently normal women during various stages of pregnancy and the puerperium. Of these 81 women, 10 were in the first trimester of pregnancy, 19 were in the second trimester, 14 were in the third trimester, 20 were at or near term and had just completed or were very near the end of labor, 8 were 3 days post partum, and 10 were 6 to 7 weeks post partum. In 15 instances fetal leukocytes were also obtained from cord blood at delivery simultaneously with maternal leukocytes and their phosphatase activities measured. In each of the 8 women during labor and the early post-partum period, repeated studies of leukocyte phosphatase activities were made during the first week of the puerperium. Also measured were enzymatic activities of leukocytes from 2 subjects with a fetus known to be dead in utero for several weeks, but in whom the placenta probably still retained some functional activity. Enzyme activities are expressed as milligrams of phosphorus hydrolyzed by 10 billion leukocytes in one hour.

RESULTS

The average leukocyte alkaline phosphatase activity in the normal nonpregnant females was 25.6 mg., quite similar to the values of 25 mg. found by

From the Departments of Obstetrics and Gynecology of the University of Texas Southwestern Medical School, Dallas, Texas, and Western Reserve University School of Medicine, Cleveland, Ohio.

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Valentine[1, 2] and 23.1 mg. reported by Wiltshaw and Moloney,[3] while acid phosphatase activity averaged 27.6 mg., differing slightly from the mean of 21.9 mg. determined by Valentine.

Fig. 1 shows the increases in leukocyte alkaline phosphatase activity associated with pregnancy. During the first trimester the mean activity was 60.4 mg. and rose to 75.0 mg. during the second trimester. The average activity during the third trimester but prior to the onset of labor was 76.5 mg. During labor at or within one hour of delivery the mean leukocyte alkaline phosphatase activity was 116 mg. while the activity of leukocytes from cord blood at delivery was 76.0 mg. Three days post partum the maternal mean leukocyte alkaline phosphatase activity increased to 213 mg. Six weeks after delivery it averaged only 35.8 mg.

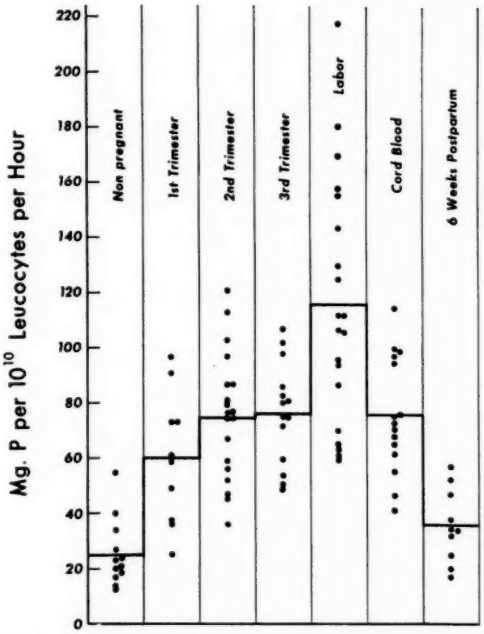

Fig. 1.—The individual and mean values for leukocyte alkaline phosphatase activity expressed as milligrams of phosphorus hydrolyzed by 10 billion leukocytes per hour are plotted for normal subjects during the nonpregnant state, first trimester, second trimester, third trimester, at the end of labor, for cord blood, and for subjects 6 to 7 weeks post partum.

Leukocyte acid phosphatase activity varied little during pregnancy and the puerperium; the mean values were first trimester 26.0, second trimester 26.2, third trimester 29.1, cord blood 23.8, 3 days post partum 27.1, and 6 to 7 weeks post partum 26.0 mg. of phosphorus liberated per hour by 10 billion leukocytes.

The average concentration of leukocytes per cubic millimeter during these periods were normal nonpregnant 7,790, first trimester 7,840, second trimester 11,350, third trimester 9,030, labor 14,100, cord blood 18,350, 3 days post partum 13,430, and 6 to 7 weeks post partum 6,480. There was no consistent

relationship between the degree of elevation of the leukocyte alkaline phosphatase activity and the blood leukocyte concentration within any of these groups.

During the immediate post-partum period following labor the leukocyte alkaline phosphatase activity rose considerably, but the degree of elevation was quite variable, ranging from 124 to 410 and averaging 213 mg. 72 hours after

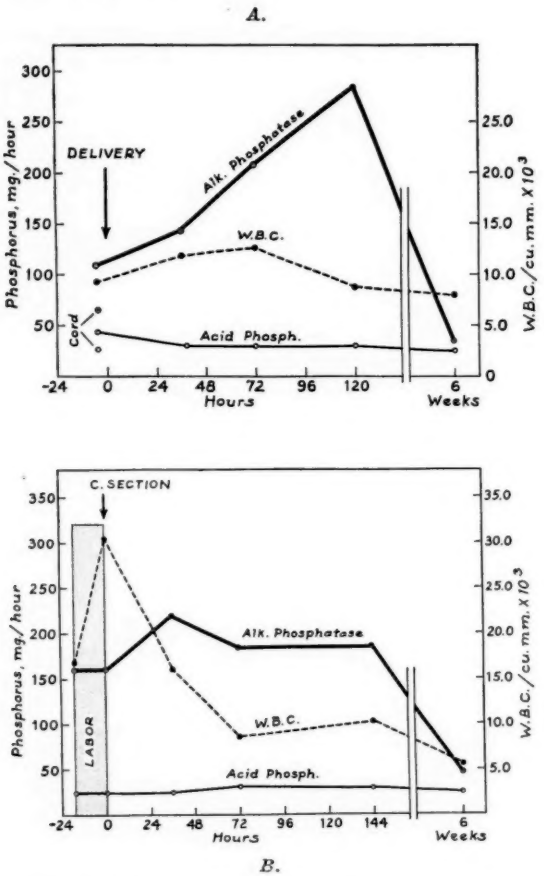

Fig. 2.—*A*, Serial post-partum leukocyte alkaline and acid phosphatase activities and white blood counts from a subject who delivered vaginally as well as cord blood and maternal leukocyte phosphatase activities at delivery are shown. While the alkaline phosphatase activity rose considerably during the early puerperium, only a slight rise in the blood leukocyte level was detected during the period studied. *B*, Data are from a patient who was delivered by cesarean section after 20 hours of labor because of cephalopelvic disproportion. A marked leukocytosis at the end of labor was not accompanied by a comparable increase in leukocyte alkaline phosphatase activity.

delivery. In some instances the post-partum enzymatic activity increased markedly but the rise was not associated with an appreciable increase in the concentration of circulating leukocytes; in other instances the opposite was true. Data from 2 representative studies are shown in Fig. 2, *A* and *B*. In Fig. 2, *A*, a normal vaginal delivery, an increase in leukocyte alkaline phosphatase activity from 111.5 just before delivery to 285 mg. 5 days later was accompanied first by a slight rise and then by a slight fall in the white blood cell

count. In the case demonstrated in Fig. 2, *B*, a cesarean section was performed 24 hours after the onset of labor because of cephalopelvic disproportion. During labor within a period of 20 hours the leukocyte count rose from 16,750 to 31,150 per cubic millimeter, but with this rather intense leukocytosis a comparable increase in leukocyte alkaline phosphatase activity was not detected.

The leukocyte alkaline phosphatase activity in cord blood was less than the maternal leukocyte activity at delivery; instead it was quite similar to that in maternal leukocytes during the third trimester, averaging 77 mg. in the former and 76.5 mg. in the latter. In only one instance was the cord blood leukocyte enzymatic activity higher than in the maternal blood obtained at the end of labor.

In one of the 2 subjects with a fetus dead in utero 8 weeks after fetal death the leukocyte alkaline phosphatase activity was 56 mg. while the leukocyte concentration was 7,080 per cubic millimeter; in the other in whom the fetus was dead for more than 4 weeks the alkaline phosphatase activity was 103.5 mg. and the leukocyte count was 13,200 per cubic millimeter.

DISCUSSION

It is apparent that during normal pregnancy there is a considerable increase in leukocyte alkaline phosphatase activity. The increase usually begins during the first trimester of pregnancy before any change in the concentration of circulating leukocytes is noted. It reaches a plateau during the second trimester and then rises rather abruptly during labor and the early post-partum period. During pregnancy and the early puerperium the degree of elevation of leukocyte phosphatase activity and the increase in blood leukocyte level frequently are not of comparable magnitude. Six to 7 weeks after delivery the leukocyte alkaline phosphatase activity approaches the normal nonpregnant level.

The mechanism by which the increase in alkaline phosphatase activity is produced is unknown. The observation of elevated leukocyte alkaline phosphatase activity in 2 subjects with prolonged intrauterine retention of a dead fetus suggests that the increase in enzyme activity is not dependent upon something elaborated by the fetus.

Valentine and associates[2] suggest a relationship between adrenal cortical activity and leukocyte alkaline phosphatase activity since the administration of corticotropin gel intramuscularly or of large doses of cortisone or hydrocortisone orally to normal subjects produces an increase in leukocyte alkaline phosphatase activity.

There is considerable evidence of increased adrenal function during normal pregnancy. The human placenta contains a material which has many of the activities of ACTH as well as the same electrophoretic pattern as ACTH.[4, 5] Gemzell[6] and Bayliss and associates[7] have demonstrated a several fold increase in 17-hydroxycorticosteroid concentration in plasma during pregnancy. A further increase is noted following labor according to Migeon and co-workers.[8]

These observations suggest but do not prove that the rise in leukocyte alkaline phosphatase activity during normal pregnancy is related at least in part to an increased production of corticoids during normal pregnancy.

SUMMARY

1. Leukocyte alkaline and acid phosphatase activities were measured during pregnancy and the puerperium.

2. Leukocyte acid phosphatase activity did not change during pregnancy and the puerperium.

3. Alkaline phosphatase activity rose early in pregnancy to reach a plateau during the second trimester and then during labor and the early postpartum period it was further elevated.

4. The mechanism which causes this increase is unknown but the possibility that it is related to increased adrenal function during pregnancy is suggested.

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EXPERIMENTAL AMINONUCLEOSIDE NEPHROSIS IN RATS

EUGENE B. FIEGELSON, M.D., JOHN W. DRAKE, M.D., AND LILLIAN RECANT, M.D.
ST. LOUIS, MO.
WITH THE TECHNICAL ASSISTANCE OF MARY B. KOCH AND NELLIE GROSSWASSER

INTRODUCTION

THE nephrotic syndrome is characterized by proteinuria, hypoalbuminemia, hyperlipemia, and edema. In man it is associated with such widely varied pathologic states as glomerulonephritis, amyloidosis, diabetes mellitus, lupus erythematosus, and the administration of the drug, trimethadione.[1] Though the fundamental lesion appears to be a renal defect with profound proteinuria, the metabolic pathogenesis of the syndrome is still obscure.

Experimental nephrosis has been produced in rats by an immunologic mechanism, namely, the injection of antirat kidney serum.[2, 3] This disease resembles glomerulonephritis, and its metabolic[4, 5] and histologic characteristics have been amply studied.[2, 3] Recently, Frenk, Antonowicz, Craig, and Metcoff[6] reported that 6-dimethylaminopurine-3-amino-d-ribose induced nephrosis in rats. It seemed pertinent to investigate this drug-induced syndrome and to compare it with the experimental immunologic disease.

This paper is concerned with a study of aminonucleoside-induced nephrosis in rats. The course of the disorder, the metabolic and histopathologic changes, as well as studies of adrenal-cortical function and response to steroid therapy are described.

EXPERIMENTAL

Immature male Sprague-Dawley rats weighing 90 to 100 grams were used. Twenty-six control animals, 7 of which were treated with cortisone, and 27 nephrotic animals, 12 of which also received cortisone, were studied. Rats were caged individually in metabolic units. After two days of adjustment to the cages and to the Purina rat-chow diet, studies were begun. Daily subcutaneous injections (0.3 ml. per 100 gram rat) of a 0.5 per cent solution of 6-dimethylaminopurine-3-amino-d-ribose* were given. Controls received injections of distilled water. Injections were continued throughout the study. In the first experiments animals were sacrificed serially from the sixth to the fourteenth day of injection, while in later studies, animals were sacrificed from the twelfth to the fourteenth day. Cortisone acetate (2.5 mg. per day) was given subcutaneously to the animals indicated from the onset of proteinuria until sacrifice. In one study control animals and steroid-treated nephrotic animals were pair-fed with the untreated nephrotics, the latter being provided with food and water ad libitum. Animals were weighed daily, and the total daily urinary protein excretion was determined by the Esbach method.[7] At the time of sacrifice, rats were decapitated and blood was collected with heparin as anticoagulant

From the Nutrition Research Laboratories, Department of Preventive Medicine, Washington University School of Medicine, St. Louis, Mo.

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for chemical determinations. Total and fractional serum proteins were determined by the Lowry microtechnique[8] using a Howe fractionation method.[9] Serum cholesterol was measured by the method of Saifer and Kammerer[10] and nonprotein nitrogen by the method of Koch and McMeekin.[11] Liver glycogen analyses were made immediately on removal of liver tissue by the microtechnique of Waalas.[12] Pooled samples of both serum and urine were subjected to paper electrophoresis, by use of a barbiturate buffer adjusted to a pH of 8.6.

Studies of adrenal cortical function were carried out. Adrenal glands obtained from control, nephrotic, and cortisone-treated nephrotic animals were dissected free of fat, weighed, bisected, and incubated in a glucose-Ringer-phosphate buffer to which one unit of ACTH had been added. At the end of a two-hour incubation in the Dubnoff shaker in an atmosphere of O_2, at 37° C., the quantitative production of steroids was measured by a spectrophotometric method described by Eisenstein.[13] Qualitative identification of these steroids was made by paper chromatography.[14] For histopathologic studies, hematoxylin-eosin, fat stains and periodic acid-Schiff stains were made of sections of kidney. Routine staining procedures were used for other organs studied.

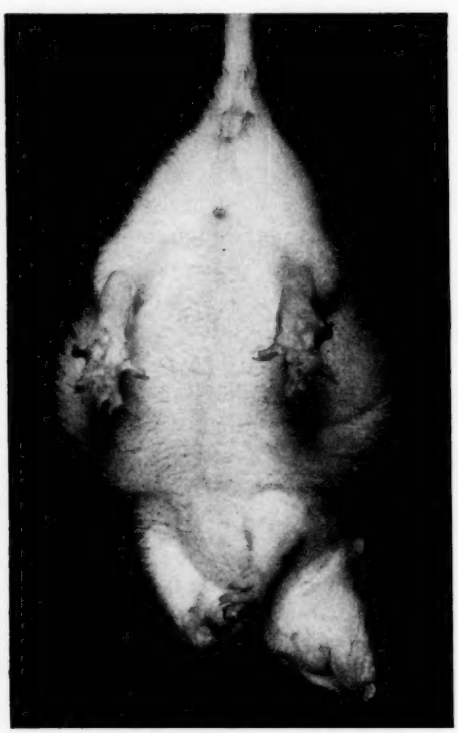

Fig. 1.—Appearance of the nephrotic rat.

RESULTS

A. *Course of the Disease.*—Aminonucleoside injections produced a nephrotic syndrome in all animals with remarkable uniformity. Proteinuria appeared as the earliest manifestation and was followed by accumulation of ascites. In Fig. 1 the characteristic appearance of the nephrotic animal may be seen. After these manifestations had appeared, cessation of injections did not alter the outcome of the disease. None of the animals survived beyond the sixteenth day.

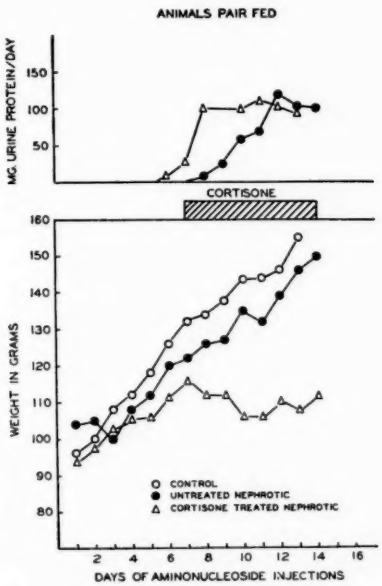

Fig. 2.—Effect of aminonucleoside injection on weight curves and proteinuria in pair-fed animals.

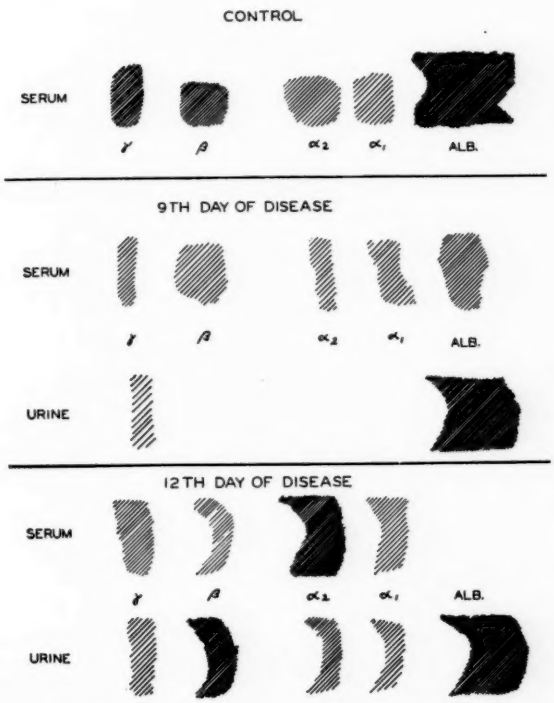

Fig. 3.—Diagrammatic representation of paper electrophoretic patterns of pooled urine and serum samples obtained from animals injected with aminonucleoside.

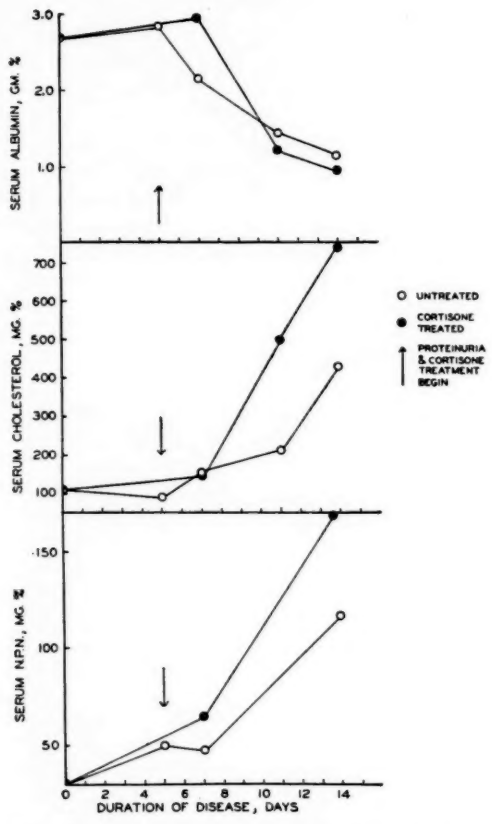

Fig. 4.—Progressive changes in serum albumin, cholesterol, and nonprotein nitrogen with injection of aminonucleoside.

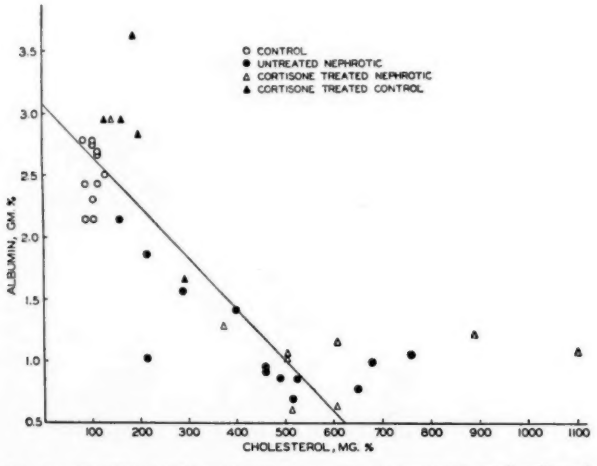

Fig. 5.—Relationship between serum cholesterol and albumin levels.

Random urine examinations revealed proteinuria, homogeneous and granular casts, and a few white blood cells. Neither red blood cells nor glucose were present.

Weight curves during the course of the disease are shown for pair-fed animals (Fig. 2). It may be seen that, despite rapidly accumulating ascites, after the tenth day, the slope of the weight curve of the nephrotic animals did not change markedly, indicating probable tissue protein losses as fluid was retained. Administration of cortisone did not favorably affect the course of the disease. A striking inhibition of weight gain was noted in steroid-treated animals, though ascites was present in every case. (Fig. 2.)

Proteinuria: Proteinuria began between the sixth and eighth day of injection, usually becoming massive by the tenth day (Fig. 2). No animal excreted less than 60 mg. protein per day. The average excretion was of the order of 100 mg. per day. Electrophoretic patterns of the urinary proteins indicated that albumin appeared in the urine first and was followed in later stages of the disease by the excretion of alpha, beta, and gamma globulins. Serial electrophoretic patterns obtained from pooled urinary samples may be seen in Fig. 3. No significant differences, qualitative or quantitative, were noted in the proteinuria of steroid-treated rats.

TABLE I

	CONTROLS	CONTROLS + CORTISONE	NEPHROTICS	NEPHROTICS + CORTISONE
Serum Albumin (Gm. %)	2.7 ± .62 (13)	2.86 ± .58 (5)	1.2 ± .46 (14)	1.2* ± .69 (9)
Total Serum Protein (Gm. %)	4.8 ± .50 (16)†	4.89 ± .63 (7)	3.24 ± .79 (14)	3.18 ± .98 (12)
Cholesterol (mg. %)	109 ± 26 (17)	162 ± 38 (7)	452 ± 175 (15)	561 ± 244 (12)
Nonprotein Nitrogen (mg. %)	27.5 ± 17 (19)	26 ± 13 (5)	115 ± 48 (13)	82 ± 91 (9)‡
Liver Glycogen (mg./Gm. wet weight)	22.2 ± 16 (19)	43.2 ± 13 (5)	8.5 ± 3.2 (9)	15.4 ± 13.4 (7)

*Mean ± standard deviation.
†Number of animals.
‡One spuriously high value included.

Hypoproteinemia: The level of serum albumin fell strikingly in the aminonucleoside injected animals following several days of proteinuria (Fig. 4). Table I shows the mean level of serum albumin and total protein in all the animals studied. It is noteworthy that cortisone did not alter the protein levels in nephrotics. In Fig. 3 the qualitative electrophoretic changes in serum proteins may be seen. Albumin levels decrease while α_2 globulin increases markedly. It is of interest that these patterns closely resemble those in human nephrosis. Similar changes occurred in cortisone-treated nephrotic rats.

Hypercholesterolemia: The concentration of serum cholesterol increased significantly as the syndrome progressed (Fig. 4). Elevations appeared to correlate with the serum albumin decrease (Fig. 5). Table I shows the mean change

noted in all the groups studied. A higher level of cholesterol was observed in all cortisone-treated animals as compared with their controls, but this difference did not prove to be statistically significant.

Serum Nonprotein Nitrogen and Liver Glycogen Levels: Progressive rise in nonprotein nitrogen without oliguria was noted in all animals treated with aminonucleoside. The levels attained may be seen in Table I.

Liver glycogen levels were uniformly lower (Table I) in the nephrotic animals and this could not be explained on the basis of deficient food intake. Cortisone significantly increased the level of liver glycogen.

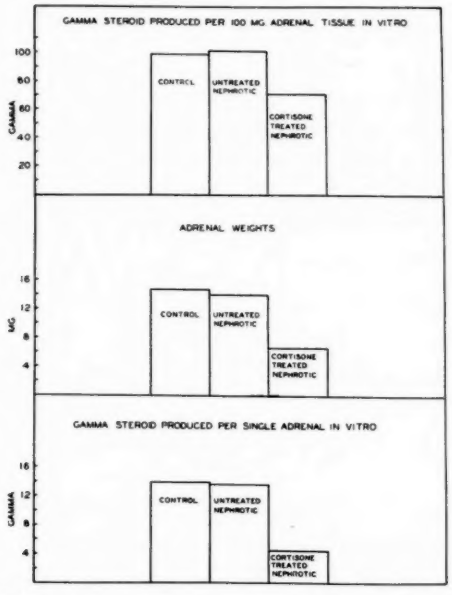

Fig. 6.—Studies of adrenal response to incubation in vitro with ACTH.

B. *Studies of Adrenal-Cortical Function in Vitro:* Six controls, six nephrotics, and four cortisone-treated nephrotic animals were studied. The adrenals were incubated in vitro with ACTH and steroid formation was measured. No significant differences in adrenal gland weight or quantitative steroid formation could be demonstrated between control and nephrotic animals (Fig. 6). Cortisone treatment of nephrotic animals resulted in a 30 per cent reduction in steroid formed per 100 mg. of adrenal tissue and a 50 per cent reduction in adrenal weight. It was of some interest that paper chromatography of the steroids produced in vitro showed no abnormal increase in aldosterone in the nephrotic group.

C. *Pathologic Studies.*—Gross: All tissues examined were found to be edematous and pale. Ascites and hydrothorax were noted. Abdominal organs appeared normal except for the kidneys. The latter were enlarged and were very pale. The capsule stripped with ease and the surfaces of the kidneys were smooth.

Microscopic: Examination of the liver, adrenal, and pancreas revealed no abnormalities. Kidney sections may be seen in Fig. 7. Nonspecific changes in the glomeruli consisting of a frayed appearance of the basement membrane and general fullness of the glomerular tuft are seen in Fig. 7, *B*. In some cases Bowman's capsule appeared distended. Casts were seen in the tubules, and in sections from those animals in the later stages of the disease (Fig. 7, *D*) much fat-staining material could be seen in tubular epithelium, endothelium, and blood vessels. It was not possible to describe basement membrane thickening in the PAS stains (Fig. 7, *C*).*

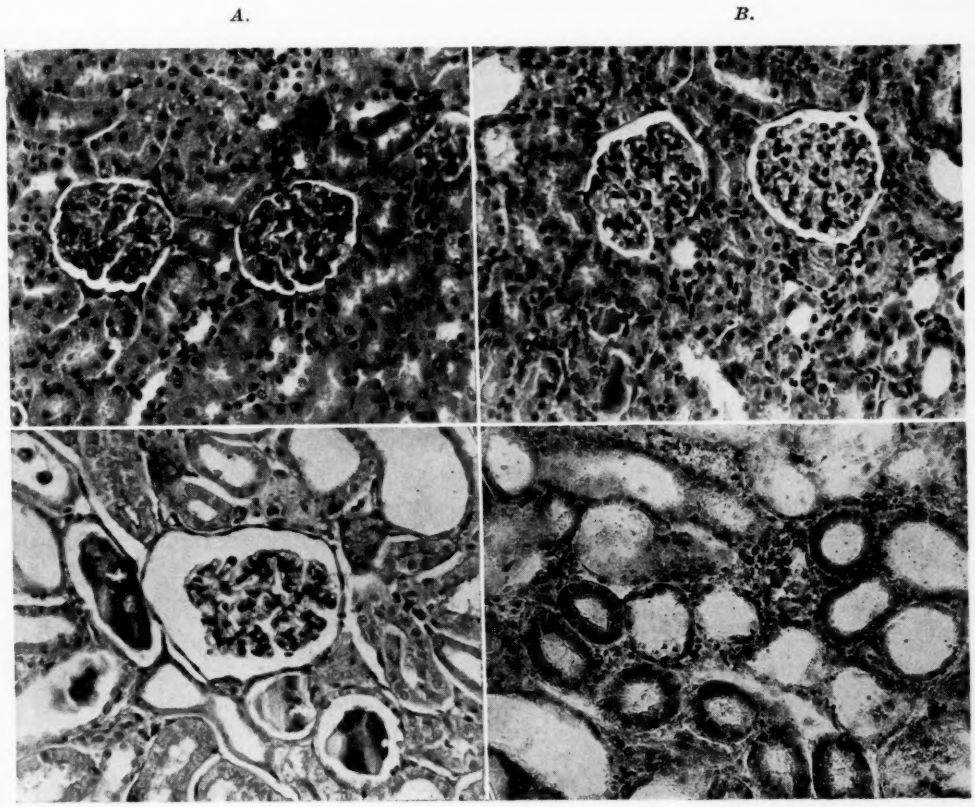

Fig. 7.—Histopathologic section of rat kidney. *A*, Control, H and E stain; *B*, nephrotic, H and E stain; *C*, nephrotic, PAS stain; *D*, nephrotic, fat stain.

DISCUSSION

The experiments described indicate that 6-dimethylaminopurine-3-amino-d-ribose is a potent nephrotogenic drug in rats. A classical nephrotic syndrome was produced over a period of 14 days. The sequence of events appeared to be massive protein loss via the kidney, starting on the sixth day, with secondary hypoproteinemia and edema. The hypercholesterolemia developed only when the

*We are indebted to Dr. W. Stanley Hartroft for interpretation of these sections.

serum proteins were significantly depressed. The development of the elevated serum nonprotein nitrogen was rather surprising in view of the minimal anatomical changes in the kidney. One is impressed that this drug-nephrosis closely resembles the antikidney serum nephrosis. The changes in the serum and urinary proteins, the hyperlipemia, and the depressed levels of liver glycogen are strikingly similar, despite the different techniques of induction. However, certain differences between these two varieties of nephrosis were observed. Rats made nephrotic with antirat kidney serum have hematuria and usually a deposition of fibrinoid material in and around the basement membrane of the glomerulus, in contrast to the drug-treated group. Perhaps the most striking difference is seen in the response to cortisone. In this study, steroid-treated nephrotic rats were adversely affected by doses of 2.5 mg. per 100 grams body weight. This was particularly notable in their weight curves. As the levels of serum protein, proteinuria, and accumulation of ascites were similar, it is assumed that the decrease in weight gain seen in the cortisone-treated group reflected a decrease in tissue protein. In the studies reported[2, 3] rats made nephrotic with antikidney serum responded favorably to the same quantity of cortisone as we used in our experiments. There occurred a decrease in proteinuria, edema, and in the basement membrane changes.

No evidence of adrenal hyperfunction in the drug-nephrosis was observed. The glands were of normal size and hypersecretion of aldosterone could not be demonstrated. This certainly does not rule out endogenous hyperaldosteronism, since these experiments were performed in vitro with exogenous ACTH added. However, Eisentein[15] demonstrated an increase in aldosterone secretion by salt-depleted rat adrenals. These data coupled with the finding of adrenal gland enlargement in the antikidney serum group,[3] as well as the difference noted with respect to cortisone response, suggest that a fundamental difference may exist in adrenal function in these two syndromes. It is not known whether such differences may be found in the variety of human nephroses. There are certainly cases of human nephrosis which do not respond to steroids. The question, however, of dosage of steroid must be raised both in the human and the animal experimental group. It is possible that larger doses might result in a favorable response.

The mechanism by which the aminonucleoside syndrome is induced presents an intriguing problem. The probability that the nucleoside produces profound enzymatic aberrations seems likely in view of the fact that there is so little evidence of morphologic changes in the kidneys and other tissues. It seems reasonable, therefore, to consider the chemical nature of this drug and to examine its potential role in metabolism.

Puromycin, an experimental antibiotic obtained from *Actinomyces alboniger*, is the parent compound of the aminonucleoside. In Fig. 8, the chemical structures of both of these compounds as well as of adenosine, a physiologically important purine, are shown. It is apparent that they both resemble adenosine and differ from each other only in that the nucleoside has a free amino

group on the ribose moeity, while Puromycin has the amino group tied up in amide linkage. Both Puromycin and the nucleoside have been reported to be effective against experimental parasitic infections (trypanasomiasis, amebiasis)[16, 17] and also against experimental tumors in mice.[18] Puromycin has been used in several cases of far-advanced human tumor.[19] Studies of the pharmacology and toxicity of Puromycin in rats indicate that the drug has a low level of nephrotoxicity.[20] Ascites and renal casts have been described only with the intraperitoneal administration of doses of 10 mg. per 100 gram rat for ten days or longer. In the experiments with the nucleoside, doses of less than 1.5 mg. per 100 gram rat produced a striking nephrotic syndrome. It seems likely

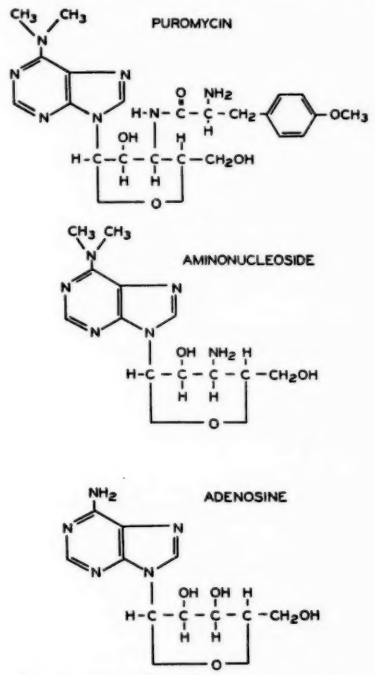

Fig. 8.—Chemical structure of adenosine, aminonucleoside, and Puromycin.

that the structural difference, namely the free amino group in the nucleoside, is in some way related to the potency of the nucleoside as a nephrotogenic agent. The similarity of both drugs to adenosine suggests that they may function as competitive analogues of adenosine. At the present time, studies of the mechanism of action of the nucleoside are under way in our laboratory.

SUMMARY

Experimental nephrosis has been produced in rats by the administration of 6-dimethylaminopurine-3-amino-d-ribose. The course of the disease, certain of the metabolic changes, and the histopathology have been described. No evidence of response to steroid therapy was obtained. The possible mechanism of action of the drug has been discussed.

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DIFFERENTIAL DIAGNOSIS OF GASTRIC ULCER AND CANCER BY A STUDY OF MAXIMALLY STIMULATED GASTRIC SECRETION

B. I. Hirschowitz, M.D., M.R.C.P., J. A. London, M.D., and
H. S. Wiggins, Ph.D.
Ann Arbor, Mich.

THE differential diagnosis of benign and malignant lesions of the stomach remains a difficult problem despite the number of diagnostic aids that are available. In any of these, the overlap between values obtained in these two conditions is sufficiently large to leave room for considerable doubt.[3] This applies particularly to the variety of gastric secretory function studies used—direct gastric analysis for a study of acid content, employing test meals, alcohol, or a single dose of histamine, and indirect gastric "analyses" using dye or quinonium exchange resins, and the plasma and urinary pepsinogen estimations. Radiology and gastroscopy and gastric cytology are superior to gastric function tests, but still fail to clearly distinguish ulcer from cancer in a moderate number of cases.

This report describes the results of an attempt to distinguish gastric ulcers from gastric cancers by employing a maximum stimulus of gastric secretion in the form of an intravenous infusion of histamine. This test has permitted the differential diagnosis between ulcer and cancer with a high degree of certainty.

METHODS

The study comprised 15 patients with cancer of the stomach, proved histologically in 13 and by gastroscopy and radiology in 2, one of whom had in addition a cancerous node in the neck proved histologically, and 16 patients with benign gastric ulcer, 3 proved histologically and the remainder followed radiologically or gastroscopically to complete healing. All had been hospitalized. The age of the cancer group was 38 to 78 years (mean 61 years), and in the ulcer group 25 to 70 years (mean 51 years).

The gastric analyses were performed in the fasting state. Levine tubes were passed orally or nasally according to the patient's preference, and in most instances were positioned fluoroscopically at the greater curve approximately opposite the angulus. Continuous suction was employed, supplemented at 15-minute intervals with syringe suction to complete consecutive 15-minute samples. After four 15-minute samples in the unstimulated (basal) state, an infusion of histamine (5.5 mg. histamine diphosphate [≡ 2 mg. histamine base] in 1,000 ml. physiologic saline to which had been added 20 mg. Benadryl to prevent side effects) was started and given at the rate of 65 to 100 drops per minute for four to eight 15-minute periods additionally. The patients flushed within a few minutes and the rate of infusion, which was not critical, was adjusted to keep the patient flushed. The Benadryl kept the patients a little drowsy. In two instances in about 120 such studies, neither of

From the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Mich.

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them in this series, minor side effects were encountered—one subject had a severe headache and one some bronchospasm. Both were relieved within 5 minutes by the additional intravenous injection of 10 mg. Benadryl. In no instance was it necessary to discontinue the test prematurely. An advantage of this form of histamine administration is that the effects of histamine (flushing and gastric stimulation) cease within 3 to 10 minutes after the infusion is stopped.

Maximal gastric secretion was apparently maintained as long as the patient was flushed (usually 60 to 80 drops per minute) and further increase to 120 drops per minute in 6 experiments caused little or no change in volume or composition of gastric juice. This steady state of maximal gastric secretion can be maintained up to 6 hours without decline of output of acid or pepsin.[1] Reproducibility: One of the cancer patients was studied on five separate occasions at two-week intervals, and though the basal secretion varied considerably, secretion under histamine stimulation produced the same maximal plateau within 2 to 3 meq. of $[H^+]$ and $[Cl^-]$ 5 ml. of volume per 15 minutes, and 100 P.U. per milliliter, on each occasion. Similar observations were made in five normal subjects, to be reported elsewhere.

Free hydrogen ion concentration ($[H^+]$) was measured by electrometric titration (endpoint pH 3.3) against 0.1 N NaOH; total chloride ion concentration ($[Cl^-]$) was measured potentiometrically employing 50 per cent $AgNO_3$ delivered from a microburette into a 2 ml. sample of gastric juice in approximately 10 ml. of ±1.0 N HNO_3. Pepsin concentration (P.U./ml.) was measured by the hemoglobin method previously described.[1] Measurements of pH were made with the sealed glass electrode.

RESULTS

The results could not be related to the age of the patient or the duration or extent of the disease.

The volumes of gastric juice per 15 minutes and the concentrations of free acid, total chloride, and pepsin are shown in Figs. 1 to 4 and in Table I. All the samples obtained in the basal state and all the samples in the stimulated state, with the exception of the first sample after starting histamine, in all 31 patients, have been included in the graphs.

TABLE I. MEAN VALUES FOR VOLUME AND COMPOSITION OF GASTRIC JUICE IN GASTRIC ULCER AND CANCER

TYPE OF SAMPLE	DISEASE	NO. OF SAMPLES	VOLUME (ML./15 MIN.)	P.U./ML.	FREE H (MEQ./L.)	TOTAL H (MEQ./L.)	TOTAL Cl^- (MEQ./L.)
	Cancer	59	17.3	387	2.2	7.4	88.6
Basal	Gastric ulcer	51	20.0	816	21.3	29.3	111.0
	% Overlap*	--	97.0	88.0	79.0	96.0	91.0
	Cancer	118	23.3	600	27.0	36.0	110.8
Stimulated	Gastric ulcer	95	36.8	1,140	102.2	109.0	151.0
	% Overlap	--	87.0	65.0	21.0	18.0	13.0

*The percentage of the total number of samples which fall in the area of overlap between ulcer and cancer indicate the chance that any one sample could have come from either group. (See also Figs. 1 to 5.)

Volume of Gastric Juice (Fig. 1).—While the 15-minute volumes of gastric secretion in the basal state were almost identical in the two diseases, the mean volume during histamine stimulation in the cancer group was only 63 per cent of that in the benign ulcer group (Table I). The overlap of values, however, was such as to make the difference in volumes meaningless for differential diagnosis.

The Concentration of Pepsin in Gastric Juice (Fig. 2).—Though the mean concentration of pepsin in the basal state was 2½ times and in the stimulated state almost twice as large in the benign as in the malignant lesions (Table I), the overlap (Fig. 2) of 88 per cent and 65 per cent, respectively, robs this measurement of any value for differential diagnosis.

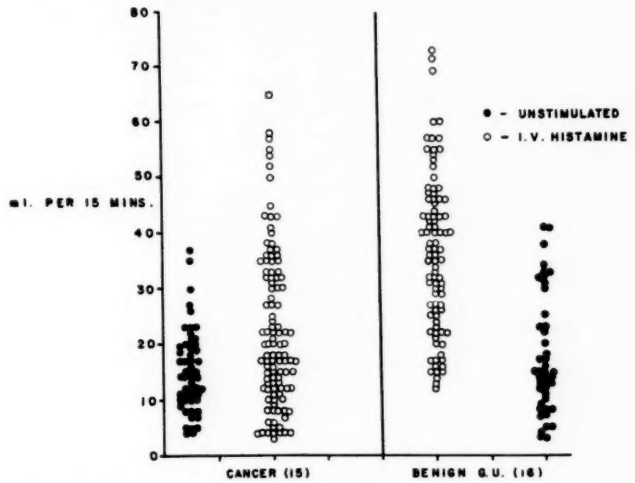

Fig. 1.—Volume of gastric juice secreted in the basal state and during histamine infusion. Each point represents one 15-minute sample.

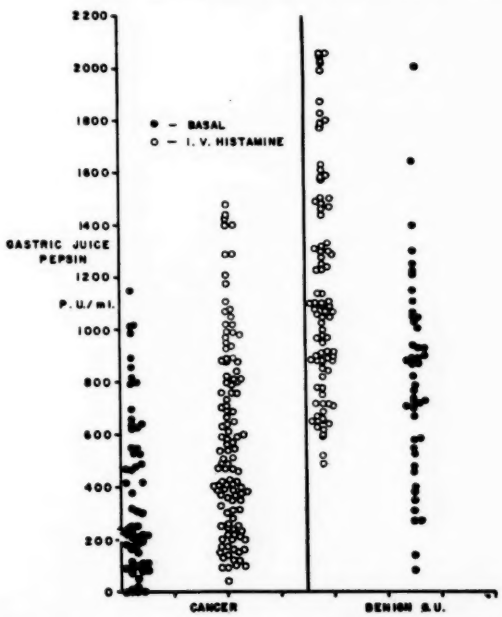

Fig. 2.—Concentration of pepsin in gastric juice (P.U./ml.). Each point represents a single 15-minute sample in the respective group.

The Concentration of Free H^+ (Fig. 3).—Differences between the benign and malignant lesions in the concentration of free H^+ were more obvious than differences in volume or pepsin concentration. In the unstimulated state, 52

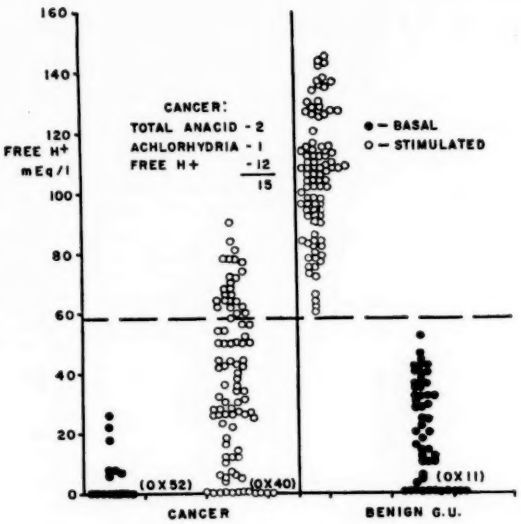

Fig. 3.—The concentration of free acid (<pH 3.3). Each point represents a single sample. In the stimulated groups the first sample after the start of histamine infusion has been eliminated.

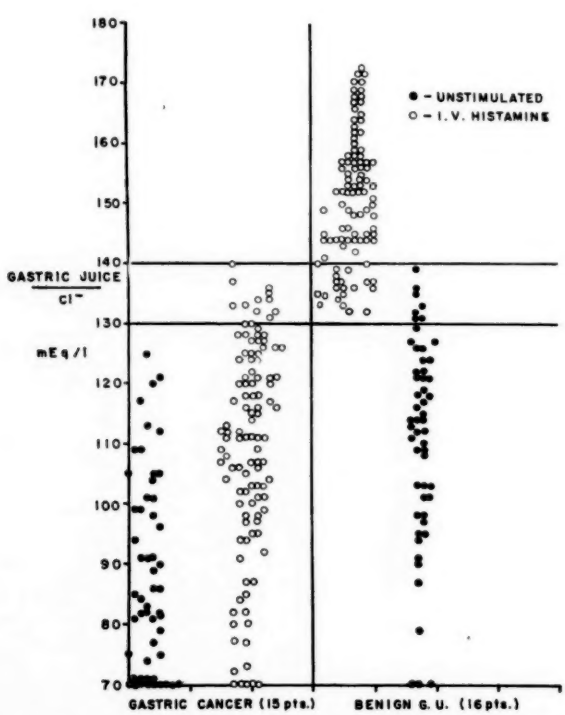

Fig. 4.—Chloride concentration in gastric juice. The normal response to stimulation is an elevation, whereas in the cancer group the chloride concentration corresponds to the range of the normal unstimulated samples.

of 59 samples contained no free acid in the cancer patients, whereas 11 of 51 samples in the ulcer group contained no free acid but the overlap was such that 80 per cent of samples could have come from either group. In the stimulated state, 40 of 118 samples contained no free acid in the cancer group and the maximum concentration reached was 90 meq./L. In the ulcer group the free acid ranged from 60 to 145 meq./L. and the overlap was 21 per cent of all samples, occupying the range of acid from 60 to 90 meq./L., leaving some room for doubt in the differential diagnosis. The maximum free H^+ concentration (Table II) was 10 meq./L. in 8 of the cancer patients; in the other 7 the maximum ranged from 60 to 90 meq./L.

TABLE II. MAXIMUM VALUES IN EACH CASE OF H^+, Cl^-, AND PEPSIN CONCENTRATION UNDER MAXIMAL STIMULATION. THE VALUES IN EACH COLUMN DO NOT NECESSARILY COME FROM THE SAME PATIENTS IN THE ORDER IN WHICH THEY ARE PRESENTED

MAXIMUM H^+/MEQ./L.		MAXIMUM P.U./ML.		MAXIMUM Cl^-/MEQ./L.	
GASTRIC ULCER	CANCER	GASTRIC ULCER	CANCER	GASTRIC ULCER	CANCER
145	90	2,800	1,480	174	140
144	84	2,300	1,420	173	136
139	81	2,300	1,300	163	134
130	78	2,300	1,210	161	133
130	77	1,830	1,150	159	132
126	52	1,630	1,080	157	130
115	60	1,460	1,000	157	126
114	10	1,320	900	156	114
112	6	1,300	760	154	113
110	6	1,280	650	153	112
108	6	1,230	570	149	112
106	4	1,140	570	148	108
104	0	1,090	410	145	106
102	0	970	360	144	100
90	0	930	250	140	82
84				139	

The Concentration of Total Chloride in Gastric Juice (Fig. 4).—The characteristic normal response of the concentration of chloride in gastric juice in man to stimulation is an increase from a level usually below 135 meq./L. in the basal state to a level between 135 and 175 meq./L. in the stimulated state. This characteristic has been observed in gastric ulcers (Fig. 4), in duodenal ulcers, and in normal subjects (unpublished observations). In sharp contrast to this response, the patients with gastric cancer, under maximal histamine stimulation, have levels of gastric juice chloride which correspond almost exactly to the levels of chloride in the normal unstimulated gastric juice (Fig. 4). The overlap between the two groups of all stimulated samples was only 13 per cent of the total.

Differential Diagnosis by Combined Use of $[H^+]$ *and* $[Cl^-]$.—In plotting the concentrations of free H^+ and total Cl^- (Fig. 5) under maximal histamine stimulation, the first 2 samples after starting histamine were excluded so as to be sure of using only unmixed stimulated samples. Of the samples from benign ulcers, all but one fell into the area of the graph defined by the lower limits

135 meq./L. Cl^- and 80 meq./L. H^+. All the samples from the cancer patients fell outside these combined limits. This allowed 100 per cent differentiation between these two conditions in the 31 patients reported here.

Achlorhydria, Anacidity, and Achylia.—If achlorhydria is defined as the absence of free but the production of combined acid (pH 3.5-6.5) and anacidity as the inability to produce any acid (pH 6.5-7.0) with or without stimulation, one patient had achlorhydria (pH 4.6) and two had anacidity, while the rest all produced free acid (pH < 3.3). However, no patient in either group had achylia (the absence of acid *and* pepsin, as in pernicious anemia), since the two patients with anacidity had pepsin levels about 10 times as great as those found in pernicious anemia.

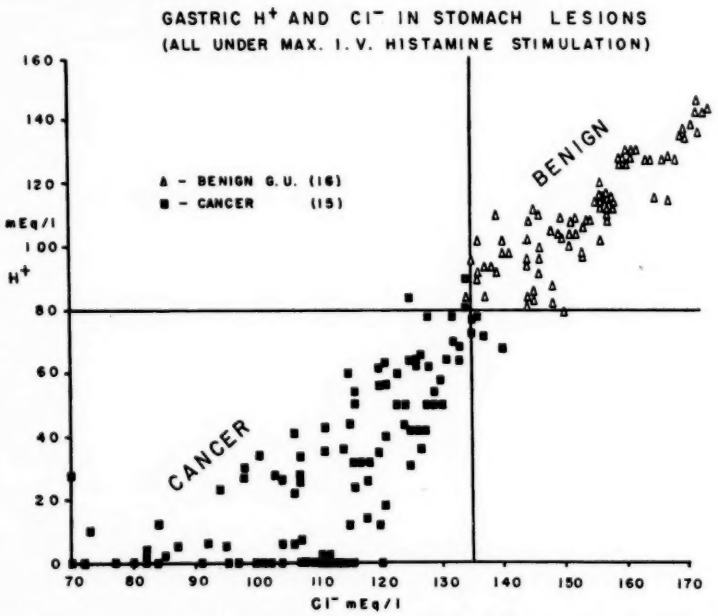

Fig. 5.—Combined plot of H+ and Cl– of all samples of gastric juice in the stimulated state with the first two after starting histamine infusion eliminated. This shows virtually complete separation between cancer and ulcer.

Exceptions to the Rule.—One patient, not included in the above descriptions, who had a bleeding duodenal ulcer of long duration and who also had carcinoma in the stomach, omentum, and liver, provided the one exception in this study. He had a 15-minute volume of gastric secretion in the basal state of 117 ml., free H^+ concentration of 134 meq./L., Cl^- concentration of 157 meq./L., and pepsin concentration of 640 P.U./ml. No further increase could be obtained with histamine stimulation. This hypersecretion in the "basal" state exceeded the values obtained in most duodenal ulcer subjects under maximum stimulation. No explanation can be offered for this phenomenon.

If hyposecretion be defined as the response to histamine which falls in the lower left-hand quadrant in Fig. 5, the same response has been found in one apparently healthy subject, in 2 patients with complete vagotomy, and in a

patient with marked malnutrition and signs of pyridoxine deficiency. No instance of an inadequate response to stimulation has been observed in any patient with benign peptic ulceration of the esophagus, stomach, or duodenum.

Histology.—While the appearance of the mucosa varied considerably, no stomach showed either advanced mucosal atrophy or extensive carcinomatous replacement. No obvious correlation between structure and function could be found. No special studies other than routine hematoxylin and eosin preparations of the specimens were undertaken.

DISCUSSION

The composition of gastric juice in the unstimulated state differs considerably from that found in the maximally stimulated stomach. Since the techniques of collection of gastric juice are such that the stomach is not entirely drained, any transient stimulation will produce a gastric juice of apparently low H^+, Cl^-, and pepsin, because of unavoidable admixture of basal and stimulated secretion. This applies especially to the single subcutaneous injection of histamine. The principal objection to the test meal, whatever its composition, is that a fixed volume of fluid of unknown chemical composition is added to a varying volume of gastric secretion. The resulting mixture, of uncertain volume because of loss through the pylorus, defies any attempt to define the true contribution of the stomach. Because of the large empirical experience, however, some standards have been obtained (Osterberg, Comfort). The physiologic knowledge gained from such studies, however, is similar to that which could have been acquired of renal function, if every urine sample were mixed with a bowl of cooked breakfast food before analysis.

These objections to the currently employed techniques for gastric secretory function studies are such as to cast doubt on the reportedly high incidence of achlorhydria in normals and in patients with gastric lesions (for a full discussion see the excellent review by Comfort), and led to the search for a technique which would supply some measure of the maximum secretory capacity of the stomach. As could have been predicted, such a test would drastically reduce the finding of achlorhydria and eliminate the low values for gastric secretion in normal stomachs. This was the finding in normals, in patients with duodenal (unpublished) and gastric ulcer, who differed only in the volume of gastric juice but not in its composition. The qualitative differences between normal and abnormal mucosae have not been fully evaluated because of the relatively small number of conditions studied, and would have to be delineated further for age and sex, for spontaneous variations in normals, and for the various disease states which affect the stomach. At present in a very limited study, the qualitative difference delineated by the chloride "threshold" of 130-140 meq./L. between normal basal and normal stimulated states has sorted out the cancerous stomach and the vagotomized stomach as hyporeactors.

Quantitative differences in volume secretion between individuals probably depend on the mass of functioning gastric mucosa, so that it can be expected that

children would have less gastric secretion than adults and that the volume would again decline in old age. This information would have to be sought in a larger group of subjects than studied here.

The test of maximal secretory capacity described here has shown no evidence of anything approaching achlorhydria in any of the 16 patients aged 25 to 70 years (mean age, 51 years) and anacidity in only 2 of 16 cancer patients. These were patients with advanced cancer, 10 being inoperable at the time of the study. From this figure of 2 patients with anacidity out of 16, one could say with 95 per cent confidence that between 2 and 40 per cent of all cancer patients would have anacidity at the time of clinically advanced cancer. Assuming the actual percentage found anacid in this study (12½ per cent) it seems unlikely that more had anacidity earlier on, since acid secretion decreases with the advance of cancer (Comfort). It is possible, therefore, to visualize a failure to predict 80 per cent of eventual cancers of the stomach if cancer surveys restrict themselves to the anacid patient. The achlorhydric patient, however, does have an 8 to 10 times greater chance of getting cancer than the patient with free acid by the tests described by Comfort. It remains to be seen whether the qualitative abnormality such as was found in cancer patients in this study precedes or follows the cancer.

The principal use of this test at present, however, is in the differential diagnosis of gastric lesions. It is probable that, as larger groups are studied, more overlap between cancers and ulcers will become evident, but from the preliminary evidence in 31 patients the test appears to offer more than the other conventional tests of gastric function.

SUMMARY

The maximum gastric secretory response to intravenously infused histamine was measured in 31 patients, comprising 15 with cancer and 16 with benign gastric ulcer. While the mean volume of gastric juice and mean pepsin concentrations differed significantly, the overlap of values between the two groups made these two measurements valueless for differential diagnosis. The concentrations of free H^+ showed less overlap and of total Cl^- even less. The combined use of H and Cl concentrations allowed differentiation between ulcer and cancer with a high degree of certainty.

By maximal stimulation of gastric secretion only 2 of 16 patients with cancer had anacidity—an incidence lower than reported found with other types of gastric analysis.

We are grateful for the technical assistance of Mrs. Esther Dullaert and Mrs. Sararuth McCool.

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DIFFERENTIATION OF POLYCYTHEMIA VERA AND SECONDARY POLYCYTHEMIA BY ARTERIAL OXYGEN SATURATION AND PULMONARY FUNCTION TESTS

JUNE M. FISHER, M.D.,* GEORGE N. BEDELL, M.D., AND PAUL M. SEEBOHM, M.D.
IOWA CITY, IOWA

THE differentiation of polycythemia vera from secondary polycythemia is sometimes a difficult task. Ratto, Briscoe, Morton, and Comroe[1] have discussed the theoretical reasons that should make this distinction possible by measuring arterial oxygen saturation. Also they point out the practical obstacles. The hypothesis is this. Uncomplicated polycythemia vera should not lead to arterial hypoxemia. Reduced arterial oxygen saturation in a patient with polycythemia should indicate polycythemia secondary to hypoxemia. This conclusion is questionable, however, for the following reasons: (1) Patients with polycythemia vera usually are more than 50 years of age, and healthy persons in this age group may have slight reduction of arterial oxygen saturation.[2] (2) Patients with polycythemia vera may have concomitant lung disease which produces arterial hypoxemia. (3) Most patients with polycythemia vera whose arterial blood has been studied have no evidence of arterial hypoxemia,[1, 3] but there are reports of arterial hypoexmia which is considered to be secondary to uncomplicated polycythmia vera.[4-6] Because of doubt about the interpretation of arterial hypoxemia in patients with polycythemia we decided to undertake the present study. This paper reports studies of arterial blood and pulmonary function in 13 patients with polycythemia vera and 9 patients with secondary polycythemia.

Patients who had a hematocrit of 60 per cent or greater were considered polycythemic. The clinical diagnosis of polycythemia vera was made on the basis of the clinical findings of leukocytosis, high platelet count, and palpable spleen in addition to polycythemia. Conditions capable of producing secondary polycythemia such as cyanotic heart disease, lung disease, high altitude, and respiratory center depression were excluded. Secondary polycythemia was diagnosed when the patient had polycythemia associated with cyanotic heart disease or lung disease in the absence of leukocytosis, high platelet count, and palpable spleen.

METHODS

The 22 patients were seen at the University Hospitals or Veterans Hospital in Iowa City. Each patient had a complete history, physical examination, and the indicated laboratory tests. The red blood cell, white blood cell, and platelet counts, hemoglobin, and

From the Pulmonary Research Laboratory, Department of Internal Medicine, College of Medicine, State University of Iowa, Iowa City, Iowa.

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TABLE I. PHYSICAL CHARACTERISTICS OF AND HEMATOLOGIC FINDINGS IN PATIENTS WITH POLYCYTHEMIA VERA

PATIENTS	AGE (YEARS)	SEX	HEIGHT (INCHES)	WEIGHT (POUNDS)	B.S.A.* M.²	RED BLOOD COUNT† (MILLION/C. MM.)	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT (%)	WHITE BLOOD COUNT (CELLS/C. MM.)	PLATELET COUNT (THOUSAND/ C. MM.)
P. B.	50	M	66	138	1.70	7.75 (5.20-10.10)	24.0 (15.1-24.0)	76 (50-81)	9,800 (9,800-16,400)	212 (98-226)
B. J.	53	F	66	136	1.68	7.96 (6.00-7.96)	22.5 (15.6-22.5)	68 (54-68)	9,850 (8,000-13,700)	316 (102-516)
G. A.	62	F	64	112	1.52	6.47 (4.68-7.22)	19.9 (13.8-19.9)	69 (47-70)	22,300 (22,300-32,250)	336 (159-453)
C. Q.	52	M	68	130	1.68	7.87 (4.45-9.53)	19.0 (13.6-19.0)	65 (49.66)	12,850 (11,000-33,000)	392 (114-710)
W. U.	63	M	72	174	1.99	6.17 (4.23-6.17)	20.4 (11.0-20.9)	70 (40-70)	10,300 (9,200-17,200)	294 (230-294)
L. W.	62	M	68	156	1.83	8.96	19.5	70 (53-70)	36,750 (36,750-58,000)	723 (723-892)
A. M.	51	F	64	143	1.68	7.00 (6.35-7.00)	16.0 (15.9-16.5)	77 (58-77)	17,900 (8,000-17,900)	830
I. M.	44	F	60	124	1.51	6.00 (5.46-8.29)	19.2 (14.0-23.4)	69 (69-71)	9,850 (5,900-13,950)	120 (88-496)
H. H.	64	M	66	130	1.64	8.65 (6.59-8.65)	18.0 (13.1-19.8)	66 (48-66)	28,700 (11,850-28,700)	314 (184-314)
L. M.	42	M	68	174	1.91	5.95 (5.50-6.88)	18.0 (16.7-20.0)	61 (53-67)	13,800 (9,400-16,100)	260 (112-344)
C. V.	69	M	64	165	1.79		15.2 (15.2-16.0)	71 (70-71)	20,600 (13,750-20,600)	162 (96-162)
M. G.	58	F	68	128	1.68	6.50 (5.13-9.04)	21.0 (15.0-21.0	64 (50-64)	6,650 (6,650-10,100)	208 (208-534)
H. B. H.	41	M	71	141	1.80	7.69 (6.00-8.00)	17.5 (16.8-20.7)	59‡	43,600 (26,800-43,600)	489

*B.S.A. = Body surface area.
†Top figure is initial blood count. Figures in parentheses represent high and low values of all counts on this patient.
‡After phlebotomy at another hospital.

hematocrit determinations were done in the clinical laboratory of the hospital. Hemoglobin was measured colorimetrically by use of a Klett-Summerson photoelectric colorimeter. Platelet counts were done by the direct method. Normal values for platelet counts in this laboratory are 100,000 to 300,000/ c. mm.

The vital capacity, inspiratory capacity, and expiratory reserve volume were measured separately by a Benedict Roth spirometer. The maximum of 3 trials was recorded. The functional residual capacity of the lungs was measured in duplicate by the nitrogen washout method of Darling, Cournand, and Richards.[7] Volumes were corrected to body temperature, ambient pressure, and saturated with water vapor, hereafter called BTPS. Rate, depth, and minute volume of ventilation were measured while the patient was breathing air, 99.6 per cent oxygen, and 7.5 per cent carbon dioxide in air. Expired volumes were collected in a Tissot spirometer simultaneously with the drawing of samples of arterial blood. Volumes were corrected to BTPS. Uniformity of intrapulmonary distribution of inspired gas was checked by the single breath nitrogen test of Comroe and Fowler[8] and the measurement of the percentage of nitrogen at the end of 7 minutes breathing of oxygen as described by Cournand, Baldwin, Darling, and Richards.[9] To measure maximal breathing capacity (MBC), the subject breathed for 15 seconds through a mouthpiece, low resistance valve, and wide tubing into a Tissot spirometer. The highest value obtained with 3 trials was calculated in liters per minute. Maximal inspiratory (or expiratory) flow rate was measured between 200 and 1,200 ml. of the inspiration (or expiration) according to the method of Danzig and Comroe.[10]

Arterial blood was obtained from the brachial artery while the semirecumbent subject breathed air and again after he had breathed 99.6 per cent oxygen for at least 10 minutes. The samples were analyzed for total oxygen content, oxygen capacity, and whole blood carbon dioxide content by the manometric technique of Van Slyke and Neill.[11] Blood was rotated with air in a tonometer at room temperature for 20 minutes before measuring oxygen capacity. Appropriate corrections were made for physically dissolved oxygen and for differences in total hemoglobin in the content and capacity samples. Direct measurement of dissolved oxygen was not made, but an approximate calculation was made on the samples collected during the inhalation of oxygen by subtracting the oxygen capacity from the total oxygen content of such blood. Arterial blood pH was measured in a closed Cambridge glass electrode at either 37° C. or, if done at room temperature, the pH value was corrected to 37° C., using the factor of Rosenthal.[12] Plasma carbon dioxide content and P_{CO_2} were determined from pH, oxygen capacity, and whole blood carbon dioxide content by use of the monogram of Singer and Hastings.[13]

RESULTS

A. *Patients With Polycythemia Vera.*—The initials, physical characteristics, and hematologic findings of the 13 patients with polycythemia vera are shown in Table I. Ten of the 13 patients had at least one red blood count above 7.0 million/c. mm. All patients had at least one white blood count above 10,000/c. mm.; and 10 of 13 had one platelet count above 300,000/c. mm. On admission 4 patients (P. B., W. U., I. M., and M. G.) had essentially normal white blood cell counts and platelet counts. In these patients the type of the polycythemia was not evident immediately. Additional studies were needed to exclude heart and lung disease and establish the diagnosis of polycythemia vera. The spleen was palpable in 9 but was not felt in 4 patients (P. B., C. Q., L. M., and M. G.).

Results of pulmonary function tests and arterial blood studies are listed in Table II. A finding of signal importance is that the arterial blood saturation of patients breathing room air averaged 96.2 per cent, ranging from 93.4 to 100 per cent. After breathing 99.6 per cent oxygen for at least 10 minutes the arterial blood saturation in all patients came to 100 per cent, and the

TABLE II. PULMONARY FUNCTION STUDIES AND ARTERIAL BLOOD STUDIES

PATIENTS	LUNG VOLUMES							MINUTE VOLUME OF VENT. (L.)	ALVEOLAR GAS DISTRIBUTION	
	VC		RV		TLC		RV/TLC × 100		SEVEN-MINUTE WASHOUT (% N_2)†	SINGLE BREATH N_2 TEST (% N_2)‡
	(ML.)	(%*)	(ML.)	(%*)	(ML.)	(%*)				
P. B.	3,400	89	2,920	120	6,320	96	46	10.0	1.0	
B. J.	3,420	100						7.4		
G. A.	2,650	87	2,830	116	5,480	100	52	7.5	1.5	
C. Q.	2,000**	48	3,420	140	5,420	82	63	15.9	0.5	
	3,020	72	3,420	140	6,440	97	53			
W. U.	4,790	96	3,720	153	8,510	115	44	8.4	2.5	2.0
L. W.	4,020	88	2,870	123	6,890	100	42	8.5	2.0	2.0
A. M.	2,970	88	2,190	90	5,160	92	42	7.7	4.0	2.0
I. M.	2,545	85	2,425	265	4,970	127	49	9.7	2.5	3.7
H. H.	3,190	78	2,160	89	5,350	84	40	7.9	1.5	
L. M.	4,870	102	2,740	229	7,610	126	36	8.6	2.5	1.5
C. V.	3,180	71	2,550	105	5,730	83	45	9.0	0.5	4.0
M. G.	3,165	94	1,650	68	4,815	88	34	10.3	0.0	0.5
H. B. H.	2,550	57	2,200	196	4,750	85	46		0.5	1.5
Mean		85		141		99	44	9.2	1.6	2.2

*% equals per cent of predicted value based on physical characteristics and age.
†Normal values for seven-minute nitrogen washout are less than 2.5 per cent N_2.
‡Normal values for single breath nitrogen test are less than 1.5 per cent N_2.
§MEFR equals maximal expiratory flow rate.

value for dissolved oxygen in arterial blood was normal in all but one patient. These patients had normal P_{CO_2} (31 to 45 mm. Hg, mean 38 mm. Hg) and normal pH (7.35 to 7.41, mean 7.39) of the arterial blood.

Two patients had definite lung disease in addition to polycythemia vera. C. Q. had a pulmonary infarct and H. B. H had a thickened calcified pleura on the left. With the exception of these two patients the vital capacities were normal. The mean vital capacity for all patients including these two was 85 per cent of the predicted normal value. The mean value for residual volume was 141 per cent of the predicted average normal. The mean RV/TLC ratio was 44. The mean total lung capacity was 99 per cent of the predicted normal value. The per cent nitrogen after 7 minutes oxygen breathing was normal in 11 of 12 patients. In 5 of 8 patients the single breath nitrogen test was slightly abnormal, but 4 of these patients were over 50 years of age. Maximal breathing capacity averaged 87 per cent of predicted value, maximal expiratory flow rate 216 L./min. and maximal inspiratory flow rate 189 L./min.

B. *Patients With Secondary Polycythemia.*—The initials, diseases, physical characteristics, and hematologic findings of the 9 patients with secondary polycythemia are shown in Table III. Four patients had at least one red blood count above 7.0 million/c. mm. and all had at least one hematocrit above 60 per cent. Seven had white blood counts persistently below 10,000/c. mm. All 8 patients on whom platelet counts were done had less than 300,000 platelets/c. mm. The spleen was not palpable in any patient.

Results of pulmonary function tests and arterial blood studies are listed in Table IV. In striking contrast to the patients with polycythemia vera not a single patient with secondary polycythemia had a normal arterial oxygen saturation while breathing room air. The values ranged from 62.5 to 90.6 per cent. All patients had definite abnormalities in the other tests depending

BLOOD STUDIES IN PATIENTS WITH POLYCYTHEMIA VERA

MECHANICAL TESTS				ARTERIAL BLOOD STUDIES					
MBC		MEFR§	MIFR‖	O_2 CONTENT REST	O_2 CAPACITY REST	O_2 SATURATION (%)		P_{CO_2} REST	pH REST
(L./MIN.)	(%*)	(L./MIN.)	(L./MIN.)	(VOL. %)	(VOL. %)	REST	100% O_2¶	(MM. Hg)	
100	100	298	267	28.17	27.76	100 + 0.43	100 + 2.42	41	7.39
81	100	107	152	25.13	25.14	100	100 + 2.60	45	7.35
68	107	191	210	17.80	18.25	97.5	100 + 1.41	35	7.40
89	91	249	158	16.85	18.04	93.4	100 + 1.71	31	7.38
100	95	334	210	25.01	26.48	94.4	100 + 1.05	39	7.38
77	78	243	211	20.43	21.26	96.1	100 + 2.19	44	7.38
76	97	128	160	23.62	25.04	94.3	100 + 1.30	36	7.37
50	67	137	152	26.17	27.30	95.9	100 + 1.91	33	7.39
85	98	201	191	19.64	20.77	94.6	100 + 0.09	38	7.39
99	81	330	330	23.19	24.27	95.6	100 + 1.89	38	7.41
47	55	187	98	22.45	23.00	97.6	100 + 1.17	35	7.37
75	84	182	146	20.29	21.02	96.5	100 + 1.80	36	7.43
99	84	215	171	19.40	20.43	95.0	100 + 1.34	39	7.40
	87	216	189			96.2		38	7.39

‖MIFR equals maximal inspiratory flow rate.

¶Values following + sign refer to ml. O_2/100 ml. blood in excess of that required to saturate hemoglobin (i.e., dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.

**Results on top line represent findings at the time the patient had chest pain, those on the next line findings 10 days later when chest pain was much improved.

on the basic disease process responsible for hypoxemia. Our patients can be grouped into 3 categories: those with pulmonary emphysema with uneven ratio of blood flow to ventilation, those with congenital heart disease with venous to arterial shunt, and one with pulmonary fibrosis with reduced diffusion across the alveolar capillary membrane.

The 6 patients with pulmonary emphysema had decreased vital capacity, increased residual volume, increase in the RV/TLC ratio, uneven distribution of inspired air, reduced maximal breathing capacity, and reduction in the maximal flow rates. The maximal expiratory flow rate was less than 100 L./min. in 5 of the 6 patients. Four of the 6 patients with emphysema had inadequate carbon dioxide elimination as well as pulmonary insufficiency for oxygenation. After the patients with emphysema breathed 100 per cent oxygen, the arterial blood became 100 per cent saturated and had normal values of 0.70 to 3.00 vols. per cent dissolved in the plasma.

The 2 patients with congenital heart disease with right to left shunt of blood had normal lung volumes, even distribution of inspired air, and normal maximal breathing capacity and maximal flow rates. They had hypoxemia on room air (81.2 and 79.0 per cent), normal P_{CO_2}, but failed to achieve 100 per cent saturation when breathing 100 per cent oxygen. The arterial oxygen saturation was 99.8 per cent in one and 93.1 per cent in the other. Since arterial saturation in these patients was not 100 per cent in addition to 2.00 vols. per cent dissolved in the plasma, some venous blood must have bypassed the lung or gone through the lung without contacting ventilated alveoli. This study could not, by itself, distinguish between an intracardiac shunt and an intrapulmonary shunt. Cardiac catheterization in Patient C. K. demonstrated a patent ductus arteriosus with severe pulmonary hypertension and

TABLE III. PHYSICAL CHARACTERISTICS OF AND HEMATOLOGIC FINDINGS IN PATIENTS WITH SECONDARY POLYCYTHEMIA

INITIALS OF PATIENTS	CLINICAL DIAGNOSIS	SEX	AGE (YEARS)	HEIGHT (INCHES)	WEIGHT (POUNDS)	B.S.A.* M^2	RED BLOOD COUNT† (MILLION/C. MM.)	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT %	WHITE BLOOD COUNT (CELLS/C. MM.)	PLATELET COUNT (THOUSAND/ C. MM.)
H. D.	Pulmonary emphysema	M	47	71	171	1.95	6.17	17.1	58	10,400	
								(15.5-18.4)	(49-62)	(6,400-14,500)	
F. S.	Pulmonary emphysema	M	49	68	143	1.75	7.47	20.0	73	8,750	80
							(5.46-7.47)	(17.0-21.0)	(56-73)	(5,450- 8,750)	(80-266)
B. S.	Pulmonary emphysema	M	53	73	268	2.42	7.07	21.0	70	7,600	48
								(16.8-21.0)	(60-70)	(7,600- 9,850)	
J. S.	Pulmonary emphysema	M	54	72	247	2.31	6.03	17.8	65	9,750	180
							(5.48-6.19)	(16.4-18.1)	(52-65)	(7,200-12,250)	(138-180)
H. B.	Pulmonary emphysema	M	59	65	150	1.70	6.91	20.4	71	6,050	120
								(17.3-20.4)	(63-71)	(6,050- 8,650)	
J. A.	Pulmonary emphysema	M	51	67	204	2.05	6.40	20.1	59	7,250	66
							(6.06-6.87)	(18.0-20.1)	(59-60)	(7,200- 7,250	(40- 66)
L. L.	Tetralogy of Fallot	M	35	64	111	1.51	7.54	21.0	65	7,550	208
C. K.	Patent ductus arteriosus	M	49	65	132	1.64	8.76	22.5	70	7,600	134
R. M.	Pulmonary fibrosis	F	52	61	120	1.51	5.98	17.4	60	5,400	184
							(5.40-6.80)	(16.6-22.7)	(58-66)	(5,400- 7,900)	(122-184)

*B.S.A. = Body surface area.

†Top figure is initial blood count. Figures in parentheses represent high and low values of all counts on this patient.

bidirectional flow through the ductus. Pulmonic stenosis, ventricular septal defect, right ventricular hypertension, and overriding aorta were demonstrated in Patient L. L. by cardiac catheterization and angiocardiograms.

One patient had pulmonary fibrosis. She had hypoxemia completely corrected by breathing 100 per cent oxygen, normal P_{CO_2}, normal vital capacity, even distribution of inspired air, and normal mechanical tests. We were not equipped to measure diffusion directly at the time this patient was studied. Chest x-rays and lung biopsy confirmed the diagnosis of pulmonary fibrosis.

DISCUSSION

We would like to emphasize the fact that our patients with polycythemia vera had arterial blood saturation of 93.4 to 100 per cent. Comroe found that normal arterial oxygen saturation in young adults is 97.5 per cent, S.D. ±2.1. This value compares with 96.5 per cent, S.D. ±1.68, for healthy subjects 50 years and older.[2] The arterial oxygen saturation in all of the patients with polycythemia vera was 93.2 per cent or greater; the difference between this and 97.4 per cent is less than 2 standard deviations. All of the patients with secondary polycythemia were below 93.2 per cent.

In 5 of 8 patients with polycythemia vera the single breath nitrogen test was slightly abnormal, but 4 of these patients were over 50 years of age. Griefenstein and associates[2] found values for this test up to 3.0 per cent in healthy persons over 50. Using this value for the upper limit of normal would allow 3 of these patients a normal value for this test.

There is disagreement about whether patients with polycythemia vera have normal or reduced arterial oxygen saturation. Our findings support those of Ratto and co-workers[1] and show that patients with uncomplicated polycythemia vera have normal arterial oxygen saturation. In all the patients with polycythemia and hypoxemia, physiologic studies demonstrated an abnormal cardiac or pulmonary mechanism which explained the hypoxemia.

Newman and associates[6] reported pulmonary function studies in 5 patients with presumed polycythemia vera. Two of their patients had arterial saturation of 69 per cent and 65 per cent, respectively. Both patients were obese and lethargic. Both had normal leukocyte and platelet counts. Limited pulmonary function studies did not explain the hypoxemia, and a diagnosis of polycythemia vera was made by exclusion. These patients may have had polycythemia vera, but this alone does not explain the hypoxemia. The hypoxemia may have been on the basis of respiratory center depression, as in the case reported by Ratto and co-workers[1]; or the patients may have had significant lung disease. The syndrome of hypoxemia, polycythemia, extreme obesity, and hypoventilation has received much attention recently.[1, 14-17] The cause of this Pickwickian syndrome is unknown. Perhaps Newman's two patients were examples of this syndrome.

Cassels and Morse[5] reported oxygen saturation, P_{CO_2} and pH of arterial blood in 17 patients with "polycythemia vera" and in 9 normal young adults. They stated that "the 17 patients had polycythemia of unknown origin without demonstrable cardiac or pulmonary disease, with one exception (a patient with scoliosis)." Seven of their 17 patients had arterial oxygen saturation

TABLE IV. PULMONARY FUNCTION STUDIES AND ARTERIAL BLO

INITIALS OF PATIENTS	LUNG VOLUMES							MINUTE VOLUME OF VENT. (L.)	ALVEOLAR GAS DISTRIBUTION	
	VC		RV		TLC		RV/TLC ×100		SEVEN-MINUTE WASHOUT† (% N_2)	SINGLE BREATH N_2TEST‡ (% N_2)
	(ML.)	(%*)	(ML.)	(%*)	(ML.)	(%*)				
H. D.	2,460	75	3,850	252	6,310	105	63	18.0	3.0	
F. S.	2,350	55						7.1		6.5
B. S.	3,180	50	2,820	116	6,000	77	43	5.1	7.5	3.0
J. S.	2,850	52	3,790	156	6,640	82	70	9.6	9.4	
H. B.	2,230	53	2,430	100	4,660	71	51	6.9	16.0	9.0
J. A.	2,100	41	2,600	107	4,700	62	55	4.6	3.0	2.2
L. L.	2,600	69	1,850	160	4,450	91	41	11.3	1.0	2.3
C. K.	2,875	70	2,900	119	5,775	88	50	7.5	1.0	
R. M.	2,330	85						11.0	1.0	1.0

*% equals per cent of predicted value based on physical characteristics and age.
†Normal values for seven-minute nitrogen washout are less than 2.5 per cent N_2.
‡Normal values for single breath nitrogen test are less than 1.5 per cent N_2.
§MEFR equals maximal expiratory flow rate.
‖MIFR equals maximal inspiratory flow rate.
¶Values following + sign refer to ml. O_2/100 ml. blood in excess of that required to saturate hemoglobin (i.e., dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.

below 93.2 per cent. Insufficient data are reported to exclude lung disease in these patients, and even if lung disease could not be identified by clinical or x-ray examination, this does not exclude it as a possible explanation for the hypoxemia.

Harrop and Heath[4] found the diffusing capacity of the lungs reduced in patients with polycythemia vera. We did not measure diffusion in our patients, but Ratto and associates[1] found normal values in 4 of their patients.

Until a basic physiologic disturbance is demonstrated in the red blood cells, the lungs, the respiratory center, or other tissue in patients with polycythemia vera, the best evidence is that arterial oxygen saturation is normal in patients with uncomplicated polycythemia vera. Arterial desaturation is evidence that polycythemia vera is complicated by or exists with another disease.

SUMMARY

We have studied the hematologic, arterial blood, and pulmonary function findings in 13 patients with polycythemia vera and 9 patients with secondary polycythemia. In those with secondary polycythemia hypoxemia was due to emphysema in 6, congenital heart disease in 2, and pulmonary fibrosis in one.

Patients with polycythemia vera usually have high white blood cell counts, high platelet counts, and palpable spleens; but this is not always so. Arterial oxygen saturation with the patient breathing room air was normal in every patient with polycythemia vera.

Patients with secondary polycythemia had elevated red blood cell counts, hemoglobins, and hematocrits in the absence of leukocytosis, increased platelets or splenomegaly. All had arterial hypoxemia. Although it was not immediately apparent in some of these patients that heart disease or lung disease was present, careful physiologic evaluation disclosed the mechanism producing hypoxemia.

Until a basic disturbance of oxygenation is demonstrated in patients with uncomplicated polycythemia vera, the best evidence is that the arterial oxygen

STUDIES IN PATIENTS WITH SECONDARY POLYCYTHEMIA

MECHANICAL TESTS				ARTERIAL BLOOD STUDIES					
MBC		MEFR§	MIFR‖	O_2 CONTENT REST	O_2 CAPACITY REST	O_2 SATURATION (%)		P_{CO_2} REST	pH REST
(L./MIN.)	(%*)	(L./MIN.)	(L./MIN.)	(VOL. %)	(VOL. %)	REST	100% O_2¶	(MM. Hg)	
62	54	73	204	21.39	24.06	89.0	100 + 1.93		
33	33	26	54	22.04	27.45	80.3	100 + 1.82	65	7.34
68	50	44	244	19.62	23.85	82.3	100 + 3.00	58	7.32
83	62	100	147	19.87	21.94	90.6	100 + 0.94	45	7.38
19	20	29	143	14.89	23.84	62.5	100 + 0.70	80	7.24
49	40	60	133	18.77	21.74	86.3	100 + 0.90	58	7.33
89	88	277	233	20.17	25.54	79.0	93.1	39	7.38
85	86	490	282	20.50	25.24	81.2	99.8	41	7.38
68	100	155	152	18.90	20.79	82.6	100 + 2.09	38	7.33

saturation is normal. Arterial desaturation is evidence that polycythemia vera is complicated by or exists with another disease.

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THE EFFECT OF MAGNESIUM SULFATE IN LOWERING TETRACYCLINE BLOOD LEVELS

ROBERT S. HARCOURT, M.S., AND MORTON HAMBURGER, M.D.
CINCINNATI, OHIO

ONE of the measures employed several years ago to counteract the gastrointestinal side effects of tetracycline drugs was the administration of aluminum hydroxide gel. It was soon discovered, however, that though it was an effective means of preventing nausea, this practice resulted in a lowering of tetracycline blood levels.[1] The possibility that the effect was part of a more general biologic phenomenon of cation antagonism of tetracycline was suggested by the later discovery that several bivalent cations antagonize the antibacterial action of tetracycline drugs in vitro.[2, 3] It seemed appropriate, therefore, to find out whether another cation which antagonizes tetracycline's antibacterial action might also lower the blood level when the cation and the antibiotic were administered by mouth simultaneously. A study was therefore undertaken in which blood levels were measured at various intervals following the simultaneous administration of tetracycline and magnesium sulfate to man and to rhesus monkeys. As a control, tetracycline was given intramuscularly and magnesium sulfate by mouth to 10 monkeys and 2 human patients. It is the purpose of this communication to report the results.

METHODS

Subjects.—Ten human beings and 20 rhesus monkeys* were studied. The humans, 7 men and 3 women, ranging in age from 39 to 60 years, were patients in the medical wards of the Cincinnati General Hospital. None of the subjects was receiving any other antibiotics. The monkeys weighed approximately 5 kilograms and were healthy. None was receiving medication.

Administration of Drug.—Oral doses of 500 to 1,000 mg. of tetracycline HCl (Achromycin, Lederle) were administered as 250 mg. capsules. Epsom salts ($MgSO_4 \cdot 7H_2O$) were given as a 50 per cent solution. The usual dose of 30 ml. (15 Gm. of magnesium sulfate) was employed except in one instance, when 15 ml. was used. In five instances 30 ml. of castor oil, a nonionic cathartic, was given instead of $MgSO_4$. In every case the magnesium sulfate or castor oil was swallowed by the subject within seconds after the tetracycline.

Tetracycline was administered to the monkeys by stomach tube (No. 18 catheter). With the tube in place, 500 or 1,000 mg. of tetracycline suspended in 20 ml. of 0.01 N HCl

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From the Infectious Disease Laboratory, Department of Medicine, University of Cincinnati College of Medicine, and the Cincinnati General Hospital.

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was introduced by syringe. The tube and syringe were then flushed with about 25 ml. of water. If Epsom salts were to be administered, 30 ml. of a 50 per cent solution was introduced immediately preceding the tetracycline.

Intramuscular doses of 200 mg. of tetracycline were given in the posterior thigh muscles of the monkeys.

In both the human beings and the monkeys, at least 48 hours elapsed between doses.

Collection and Handling of Blood Samples.—Ten milliliters of blood was drawn from the patients by venipuncture immediately before a dose and at 1 and 4 hours after the dose. The blood was placed in sterile centrifuge tubes and allowed to clot at room temperature. The samples were centrifuged and the clear serum drawn off and stored at 1° to 3° C. until assayed.

Samples from monkeys were also collected by venipuncture. Those receiving tetracycline by stomach tube were sampled just before the dose and at 2 and 3 hours after the dose. Those receiving intramuscular tetracycline were bled just prior to the dose and at 1 and 2 hours.

Method of Assay.—Each serum sample was assayed twice. The first assay was by a twofold serial dilution method, employing *B. cereus* as the test organism.

After the approximate antibiotic concentration in the serum had been determined, another aliquot was assayed by means of a measured dilution method in order to determine the level more precisely. Five-tenths milliliter of appropriately diluted serum was placed in the first tube, 0.45 ml. in the second, 0.40 ml. in the third, etc. The tenth tube contained 0.05 ml. The volumes were adjusted to 0.50 ml. with broth and the tubes inoculated, incubated, and read after 18 hours' incubation at 37° C. A control solution of tetracycline of known strength was run with each assay.

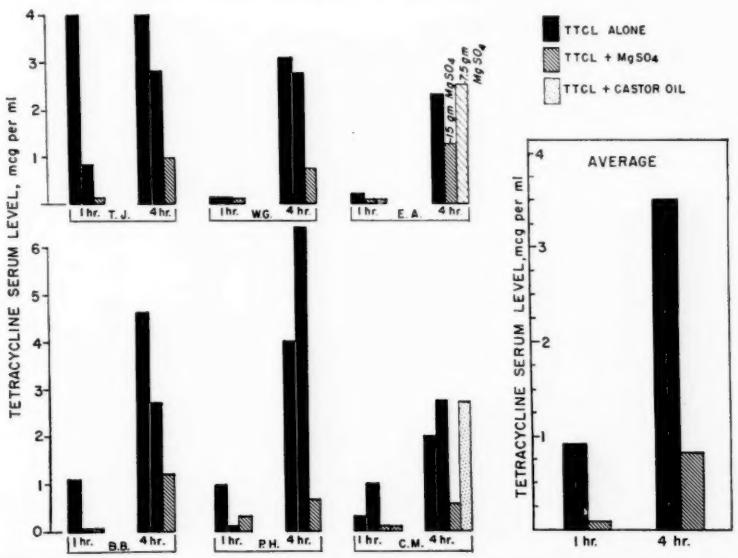

Fig. 1.—Tetracycline (TTCL) serum levels in human subjects following administration of 500 mg. tetracycline HCl by mouth, with and without simultaneous magnesium sulfate.

RESULTS

Lowering of Blood Level by Magnesium Sulfate Administered Simultaneously.—Six human subjects were given 500 mg. of tetracycline alone and, a few days later, the same dose followed immediately by 15 Gm. of Epsom salts. Two separate sets of levels after administration of tetracycline alone were determined in 5 of the patients. In every case except one the blood level when magnesium

sulfate was administered was lower at the end of 1 hour than when the tetracycline was given alone. By the end of 4 hours the tetracycline levels were uniformly lower when Epsom salts were given. The magnitude of the lowering was approximately fourfold, i.e., from an average of 0.8 to 0.2 mcg. at 1 hour

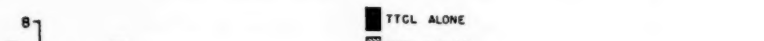

Fig. 2.—Tetracycline (TTCL) serum levels in human subjects following administration of 1,000 mg. tetracycline HCl by mouth, with and without simultaneous magnesium sulfate.

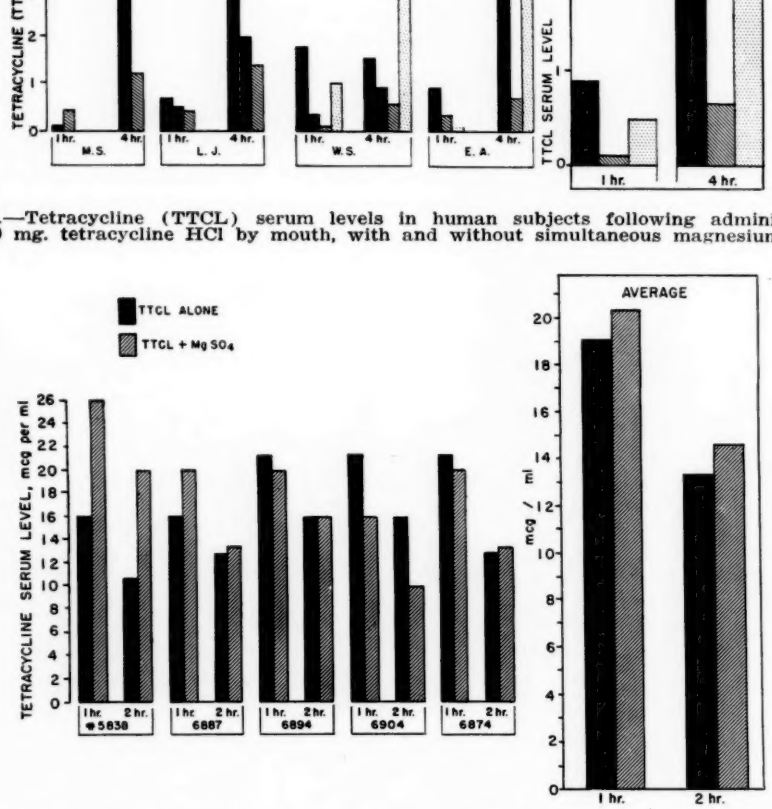

Fig. 3.—Tetracycline (TTCL) serum levels in monkeys following administration of 200 mg. tetracycline HCl intramuscularly, with and without simultaneous magnesium sulfate.

and from 3.5 to 0.8 mcg. at the end of 4 hours. The experiments are shown in graphic form in Fig. 1. Because of the possibility that the lowering of the blood level might have been due to flushing associated with the catharsis, one patient was given a dose of castor oil along with the tetracycline. The castor oil produced no lowering of blood level. Four patients were given 1,000 mg.

of tetracycline, with and without magnesium sulfate or castor oil. The results, as shown in Fig. 2, were essentially the same as with the smaller dose. A similar lowering of the blood level was found when the study was repeated in rhesus monkeys. The lowering effect was found in all of 10 animals studied with a 500 mg. dose and all of 5 studied with a 1,000 mg. dose.

In order to test whether the effect took place in the gastrointestinal tract 5 monkeys were given tetracycline intramuscularly, followed in a few days by tetracycline intramuscularly and magnesium sulfate by stomach tube. As shown in Fig. 3, magnesium sulfate by mouth did not lower the blood level when tetracycline was given intramuscularly. A similar study was carried out in 2 stuporous hemiplegic human patients, employing a dose of 500 mg. of tetracycline intramuscularly. Here too, there was no diminution in tetracycline blood level.

DISCUSSION

These observations clearly demonstrate that when magnesium sulfate is administered concomitantly with tetracycline given by mouth, the blood level of the antibiotic is suppressed approximately fourfold as compared with the level when no magnesium is given. This effect is abolished when tetracycline is given intramuscularly and the magnesium by mouth. Whether the effect is dependent upon the failure of absorption of the tetracycline or whether it is explained by the absorption of an inactivated tetracycline is not known. Two British chemists, Albert and Rees,[4] have stated that when tetracycline and aluminum hydroxide gel are mixed, a complex is formed which is not absorbed, though they presented no clinical data to substantiate their in vitro chemical experiments. Magnesium sulfate itself is very poorly absorbed from the gastrointestinal tract. The normal blood level of magnesium for human beings is 1.8 to 2.4 mg. per cent, as determined by flame photometry on patients in the Cincinnati General Hospital by Dr. James Agna.[5] Dr. Agna, in 3 separate determinations on patients given 15 Gm. of magnesium sulfate by mouth, could find no evidence at the end of 1 and 4 hours of any increase in magnesium blood levels. These data do not, however, exclude the possibility that a magnesium tetracycline complex might be absorbed into the blood stream.

In our studies the ratio of magnesium to tetracycline in terms of molecular weight was approximately 60 to 1. In one patient where levels were compared employing a smaller dose of magnesium sulfate, giving a magnesium-tetracycline ratio of 30 to 1, there was no diminution in tetracycline blood levels. Magnesium ion inactivates the antibacterial effect of tetracycline against several species but the ratio of magnesium to tetracycline required to produce this effect is much higher than that observed in our absorption studies. Thus, 2,000 atoms of magnesium antagonize the action of 1 molecule of tetracycline when *Pseudomonas aeruginosa* is used as a test organism,[3] 3,000 magnesium to 1 tetracycline when *Staphylococcus aureus* is used,[6] and 50,000 to 1 when *E. coli* is used.[2] Contrariwise, the chemical avidity of magnesium for tetracycline is said by

Albert[7] to be 1:1 or 1:2, depending upon the pH. It seems evident that magnesium is capable of antagonizing tetracycline, probably by combining with it in some way, but that the ratio of the two substances varies with the conditions under which the reaction takes place.

SUMMARY

When tetracycline and magnesium sulfate were given simultaneously to man or rhesus monkeys, the blood levels of tetracycline were found to be only about 25 per cent of those when the antibiotic was given alone. When tetracycline was given intramuscularly and magnesium sulfate by mouth, no lowering of the blood level occurred. Also, when castor oil was substituted for magnesium sulfate in the oral absorption experiments, there was no lowering of the tetracycline blood level.

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LABORATORY METHODS

A SPECIFIC ENZYMATIC ASSAY FOR THE DIAGNOSIS OF CONGENITAL GALACTOSEMIA

I. The Consumption Test

Elizabeth P. Anderson, Ph.D.,* Herman M. Kalckar, M.D., Ph.D., Kiyoshi Kurahashi, Ph.D.,** and Kurt J. Isselbacher, M.D., Ph.D.***
Bethesda, Md.

CONGENITAL galactosemia is a hereditary disease of childhood characterized by an inability to metabolize galactose. It has been found that the blood and liver of patients with this disorder lack a specific enzyme essential for galactose metabolism. This enzyme, called galactose-1-phosphate uridyl transferase, or briefly Gal-1-P uridyl transferase,[1-4] catalyzes the following reaction involving uridinediphospho-glucose†:

$$\text{I. Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal.}$$

The presence or absence of this enzyme in blood can be determined easily; this forms the basis of an enzymatic assay, described in the present report, which can be used to diagnose cases of congenital galactosemia. Such a test eliminates the necessity of subjecting these patients to the deleterious effects of galactose ingestion in a tolerance test.

Human erythrocytes contain a considerable amount of Gal-1-P uridyl transferase. The UDPGal formed in this reaction accumulates without being converted by UDPGal-4-epimerase,[1] formerly called galacto-waldenase,[5] into UDPG. This is due to the fact that hemolysates require the addition of a considerable amount of DPN before the 4-epimerase present becomes active.[6] In the absence of galactose-1-phosphate, UDPG incubated with hemolysates over a period of 30 to 60 minutes is consumed to only an insignificant degree. If galactose-1-phosphate is added in considerable excess over UDPG, most of the UDPG is consumed (i.e., converted into UDPGal). This is partly due to the fact that G-1-P, the other reaction product, is almost completely converted into glucose-6-phosphate by the phosphoglucomutase also present in hemolysates.

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Md.
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*Fellow in Cancer Research of the American Cancer Society.
**Fellow of the Jane Coffin Childs Memorial Fund.
***Massachusetts General Hospital, Biochemical Research Department, Boston 14, Mass.

†The following abbreviations are used: UDPG for uridinediphospho-glucose, UDPGal for uridinediphospho-galactose, UDPGA for uridinediphospho-glucuronic acid, DPN for diphosphopyridine nucleotide, DPNH for reduced diphosphopyridine nucleotide, Gal-1-P for α-galactose-1-phosphate, G-1-P for α-glucose-1-phosphate.

If inorganic pyrophosphate (PP) is added to UDPG incubated with hemolysate there is also a large consumption of UDPG. This is due to the following reaction in which uridine triphosphate (UTP) is formed:

$$\text{II. PP + UDPG} \rightleftharpoons \text{G-1-P + UTP.}$$

This enzyme is called UDPG pyrophosphorylase.

Reactions I and II are both measured as the consumption of UDPG in the complete incubation mixture compared with the consumption in hemolysates when UDPG is incubated without the addition of pyrophosphate or galactose-1-phosphate. UDPG is determined in heat-inactivated hemolysates by means of a highly specific dehydrogenase[7] which catalyzes the following reaction:

$$\text{UDPG + 2 DPN} \rightarrow \text{UDPGA + 2 DPNH.}$$

The increase in optical density at 340 mμ brought about by the reduction of DPN in this manner is a measure of UDPG. It should be emphasized that UDPGal does not reduce DPN in this reaction. Since 2 moles of DPN are reduced for each mole of UDPG present, the reduction constitutes a particularly sensitive analytic method. Thus, 0.01 μM of UDPG per ml. gives a change of 0.120 on the logarithmic scale of the Beckman DU ultraviolet spectrophotometer and can be readily determined with an accuracy of about ±10 per cent.[8]

It has been found that hemolysates from humans are rich in enzymes catalyzing reactions I and II.[3, 6] In human congenital galactosemia, however, the enzyme "PGal transferase," catalyzing reaction I, is absent in hemolysate or present in amounts less than 5 per cent of the normal levels.[2, 3] The other enzymes, including the UDPG pyrophosphorylase, catalyzing reaction II, are present within normal range.[3, 6] Hence, if hemolysates are found devoid of the enzyme catalyzing step I, but showing a normal activity with respect to the enzyme catalyzing step II, congenital galactosemia should be strongly suspected. If both enzymes are missing, it is most likely due to improper handling of the blood sample or the hemolysate. The test should then be repeated on another blood sample from the subject.

MATERIALS AND METHODS

Stock Solutions.—

1. Buffers: (a) Tris (tris [hydroxymethyl] aminomethane),* 0.2 M, brought to a pH of 8.1 by means of 1 N HCl. (b) Glycine, 1 M, brought to a pH of 8.7 by means of 5 N NaOH.

2. α-Galactose-1-phosphate, Monobarium salt decomposed with 10 per cent Na_2SO_4; barium sulfate precipitate washed with H_2O and the washwater added to the supernatant, 7.5 μM/ml.,† pH ca. 7.5.

3. Inorganic pyrophosphate, 0.05 M, brought to a pH of ca. 7.5 by means of 1 N HCl.

4. UDPG, disodium,‡ 5 μM/ml., pH ca. 7.5.

5. Cysteine, 210 μM/ml.; neutralized immediately before use to pH 7 or 8 with 1 N NaOH. (Use pH test paper.)

6. DPN, free acid,‡ 50 μM/ml., brought to pH ca. 8.0 by means of 0.1 N NaOH.

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*Obtainable from G. Frederick Smith Chemical Company, 867 McKinley Avenue, Columbus, Ohio.

†Commercial preparation to be available from Sigma Chemical Company, 4648 Easton Avenue, St. Louis, Mo.

‡Obtainable from Sigma Chemical Company, St. Louis, Mo., and Pabst Laboratories, Milwaukee 3, Wis.

7. UDPG dehydrogenase prepared according to Strominger and associates.[8] This enzyme preparation should contain 3,000 to 5,000 units per mg. protein. Keep at −15° C. as a solution containing 3 to 5 mg. per milliliter. Do not lyophilize.*

Special Equipment.—(a) Microvolumetric pipettes, Linderstrøm-Lang-Holter.† (b) Fused silica cells for Beckman DU spectrophotometer, capacity 1.5 ml., dimensions: 50 mm. high, 5 mm. wide and 10 mm. lightpath.‡

Preparation of Blood Sample.—Venous blood (in amounts ranging from 2 to 8 ml.) is drawn and put into a 12 ml. heavy-duty Pyrex centrifuge tube containing heparin. The blood is mixed with the heparin by inversion of the tube, the opening being covered with "parafilm." The tube is then cooled by being placed in cracked ice. The blood is centrifuged in the cold at 600 × g (full speed, 2,000 r.p.m., in an International Clinical centrifuge) for 15 minutes and plasma is aspirated off by means of a pipette with a fine tip which is slightly bent. The red cells are washed twice with a double volume (i.e., two times the volume of the packed red cells) of cold 0.9 per cent sodium chloride and the saline is pipetted off each time in the same way. In order to remove the saline as thoroughly as possible, it is advisable to sacrifice the upper layer of erythrocytes. The washed red cells are finally hemolyzed by the addition of an equal volume (i.e., the volume of the packed red cells) of distilled water. Pyrex- or quartz-distilled water is preferable. If the hemolysate is transparent, the hemolysis is complete or close to completion. Freezing and thawing the hemolysate helps to make the hemolysis complete and does not decrease the activity of the enzyme to be assayed to any detectable degree.

Estimation of Red Cell Material as Expressed as Hemoglobin.—The amount of cell material present in the hemolysate can be estimated by a hemoglobin determination which is carried out as follows[10]: 20 μl of the hemolysate (collected with a micro volumetric pipette) are blown into 5 ml. of dilute ammonia (4 ml. concentrated ammonium hydroxide to 1 L. H_2O); the solution is then mixed, shaken in air to saturate it with oxygen, and read in the Beckman spectrophotometer at 540 mμ.§ The reading on the logarithmic scale (density scale) times the molecular extinction coefficient of hemoglobin at 540 mμ (14,800)[11] multiplied by the molecular weight (16,700)[11] gives the concentration of hemoglobin in milligrams in the diluted sample. Since the sample was diluted 250 fold and since the enzymatic activity is expressed in micromoles substrate converted per hour per gram hemoglobin, the reading at 540 mμ (D_{540}) should be multiplied with the following factors in order to obtain the hemoglobin in grams per milliliter undiluted hemolysate:

$$D_{540} \times \frac{1}{14{,}800} \times 16{,}700 \times 250 \times \frac{1}{1{,}000} \text{ or } D_{540} \times 0.282.$$

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*Commercial preparation to be available from Sigma Chemical Company, 4648 Easton Avenue, St. Louis, Mo.

†Appropriate for this purpose is a selected constriction pipette obtainable from Mr. Pedersen, Carlsberg Laboratorium, Copenhagen, Valby, Denmark, or the Arthur H. Thomas Company, Philadelphia, Pa. (No. 2464-H for 50-300 μl, and No. 2464-J for 5 to 40 μl, pp. 185-186, 1950 catalog). See also reference 9.

‡Obtainable from the Pyrocell Manufacturing Company, New York, N. Y.

§If utmost accuracy is needed and especially if the hemolysis for one reason or another is not complete, the concentration of hemoglobin released from erythrocytes can be estimated in the following way. To a 1 ml. aliquot of the hemolysate add 0.1 ml. 10 per cent NaCl and mix. Spin about 30 minutes at 5,000 to 8,000 r.p.m. (For instance, in the high speed attachment head of the International PR-2 refrigerated centrifuge.) Take 20 μl of the supernatant and blow over into 5 ml. 0.2 N ammonia and read at 540 mμ for hemoglobin determination. The value found here represents the best expression for soluble erythrocyte material. This hemoglobin value may be significantly less than that of the sample taken directly from the hemolysate. However, usually the fraction of nonhemolyzed cells is so small that no special precautions of the kind described here are warranted. It should also be noted that whole blood (heparinized) can be used directly since the plasma does not contain any of the enzymes. In this case the blood (fresh or stored for 1 to 2 days at 0° to +2° C.) is first completely frozen by rotating the tube in a mixture of dry ice and ethanol. It is then completely thawed at 37° C., once more frozen, and once more thawed. The hemolyzed blood is then placed in crushed ice and a 20 μl sample is drawn for hemoglobin determination as described for the red blood cell hemolysate (see main text). Hemolyzed whole blood or red blood cells can be stored in a freezing compartment at −10° C. for a period of several days without significant loss in the activity of the enzyme. If whole blood is being used, the deproteinization should be performed with alcohol and chloroform as described previously[3] and not by heating since the latter procedure in this case does not give clear filtrates.

Incubation.—Centrifuge tubes, 12 ml. heavy-duty Pyrex, are recommended as incubation tubes. A mixture for use in all three samples is made with the following composition: 1 ml. tris buffer plus 0.1 ml. UDPG stock solution. Exactly 0.3 ml. (a constriction pipette* of 300 μl volume is very suitable for this purpose) of this mixture is placed in each centrifuge tube. To sample 1 is added 0.05 ml. galactose-1-phosphate. To sample 2 (control sample) is added 0.05 ml. distilled water. To sample 3 is added 10 μl pyrophosphate stock solution plus 0.25 ml. distilled water. Finally, the hemolysate is added as follows: 0.25 ml. to samples 1 and 2 and only 0.05 ml. to sample 3. Less hemolysate is added to sample 3 because of (a) the high activity of the pyrophosphorylase and (b) the tendency in this sample for liberation of hemin pigment into the filtrate during deproteinization, making spectrophotometric readings too high. The samples are incubated at 37° C. for 30 minutes.

Deproteinization.—At the termination of the incubation period one milliliter of distilled water is rapidly added and the mixture is heated at 100° C. for 2 minutes with rapid and constant stirring with a glass rod. The sample is then cooled in ice and centrifuged. The supernatant is aspirated off for the assay by means of a large constriction pipette. It may be necessary to centrifuge the supernatant twice in order to obtain a clear filtrate.

An alternative but somewhat more elaborate method, employing chloroform-ethanol for deproteinization, and a dual analysis with both UDPG dehydrogenase and UDPGal-4-epimerase, have been described previously.[3] The method described here, however, should be fully adequate for routine determination.

Assay.—An aliquot of 0.3 ml. of the clear supernatant is transferred with a constriction pipette to a Beckman cuvette.† The following additions are then made to the sample: 0.15 ml. glycine buffer, 0.03 ml. neutral cysteine solution, 0.02 ml. DPN., 0.5 ml. distilled water, and finally 10 μl UDPG dehydrogenase (= 80 units).‡ The latter is first added to the control, the sample mixed, and the reaction followed in this cuvette.§ The mixing of the assay mixture with the enzyme is effectively accomplished by placing a piece of parafilm on the top of the silica cuvette and inverting rapidly several times. It is particularly important to obtain readings at 340 mμ as early as 20 or 30 seconds after the addition of the enzyme and subsequently at 20- to 30-second intervals during the first 2 minutes after the enzyme addition in order to be able to extrapolate back to the reading at zero time. At 2½ minutes the density at 400 mμ is read, and at the termination of the assay another reading at this wave length is made; this is done in order to correct for nonspecific density changes brought about by variations in turbidity or other extraneous factors. The readings at 340 mμ are recorded at 6 and 10 minutes and until the density increase signifying reaction has leveled off. This usually requires 20 to 30 minutes; some nonspecific increase in density may continue in both samples using certain dehydrogenase preparations, but this may be considered negligible if it is less than 0.005 density scale units per minute. Since the initial readings (up to 3 or 4 minutes after the start) and the terminal readings are the crucial ones, it is possible after starting the reaction in the first sample to add UDPG dehydrogenase to the second cuvette 4 to 6 minutes later and likewise 4 to 6 minutes after the start of the second cuvette to add enzyme to the third cuvette. The same routine described for the first sample is carried out for the second and third. Since a 0.3 ml. aliquot corresponds to one-fifth of the entire incubate, the values obtained refer to a volume of 50 μl hemolysate.

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*Obtainable from Mr. Pedersen, Carlsberg Laboratorium, Copenhagen, Denmark, or the Arthur H. Thomas Company, Philadelphia, Pa.

†Obtainable from the Pyrocell Manufacturing Company, New York, N. Y.

‡One unit is the amount of enzyme which produces a Δ_{340} of 0.001 per minute in 0.5 ml.[3] Sufficient dehydrogenase is used to give a Δ_{340} of approximately 0.040 per minute in 1 ml. In our preparations this is 0.01 ml., but if the enzyme preparation used is less active than this, part of the water in the assay can be replaced by additional dehydrogenase. Care should be taken when adding the enzyme so that the droplet which is delivered above the surface of the mixture in the cuvette is not blown into foam. It is preferable to leave a trace in the pipette in order to avoid this.

§The density at zero time at 340 mμ usually amounts to 0.30 to 0.40. However, the cuvette containing the filtrate from the sample incubated with inorganic pyrophosphate often shows a density which is somewhat higher (0.50 to 0.70).

Calculation of Gal-1-P Uridyl Transferase Activity.—The difference in ΔD_{340} (increase in optical density at 340 mμ) between the blank and the samples containing galactose-1-phosphate or pyrophosphate constitutes the basis for the calculation of UDPG consumption.* In order to express the value in micromoles substrate converted per hour per Gm. hemoglobin, it is first necessary to transform the density differences at 340 mμ (ΔD_{340}) into micromoles. By the method used 1 micromole per ml. converted would correspond to a ΔD_{340} of 12.0.[8] The micromoles UDPG converted in 1 ml. hemolysate in 1 hour would be: $\Delta D_{340} \times 20 \times \frac{1}{12.0} \times 2$ or $\Delta D_{340} \times 3.3$. As mentioned above, the amount of hemoglobin, expressed in grams, in 1 ml. hemolysate is derived from the reading of the dilute aliquot at 540 mμ multiplied by 0.282. The enzyme activity expressed in micromoles substrate converted per hour and per Gm. hemoglobin is therefore obtained from the following equation:

$$\frac{\Delta D_{340} \times 3.3}{D_{540} \times 0.282} \text{ or } \frac{\Delta D_{340}}{D_{540}} \times 11.7.$$

Calculation of UDPG pyrophosphorylase activity is similar except that the formula is multiplied with a factor of 5 since only one-fifth as much hemolysate is used.

TABLE I. ACTIVITY OF PGAL URIDYL TRANSFERASE AND UDPG PYROPHOSPHORYLASE IN RED BLOOD CELL HEMOLYSATES
(Rates expressed as uridyl transfer, micromoles per ml. red blood cells per hr., 37° C.)

PATIENT	PGal TRANSFERASE	UDPG PYROPHOSPHORYLASE
J. W., 6 yr., M., normal	1.80	3.75
J. R., 7 mo., M., normal	1.35*	3.60
A. F., 6 yr., M., normal	2.61	2.46
T. F., 8 yr., M., normal	2.61	
K. F., 7 mo., F., normal	3.00	
B. M., 4 yr., F., normal	>2.25	
B. M., 3 yr., M., normal	2.61	
H. K., 47 yr., M., normal	3.90	
V. McC., 5 mo., M., milk allergy	1.80*	
M. B., 7 mo., M., milk allergy	2.25*	
C. M., 13 mo., M., milk allergy	3.00	4.44
N. J., 6 yr., M., nephritis	2.70	
M. L., 24 yr., F., chorion epithelioma metastic (+ lactation)	2.76	
A. B., 20 yr., M., postnecrotic liver cirrhosis	2.50	

*Reaction run in the reverse direction, i.e., G-1-P + UDPGal ⇌ Gal-1-P + UDPG.

RESULTS AND DISCUSSION

Transferase Values as Related to Congenital Galactosemia.—The values for the activity of PGal transferase in erythrocytes have been determined in a large number of both male and female subjects of various ages and on various diets; normal subjects as well as subjects afflicted with various diseases have been studied.[3] Expressed per Gm. hemoglobin, the average value was found to be of the order of magnitude of 2.5 to 3.5 μM (see Table I). The normal variations are moderate, amounting to about ± 30 per cent. It is, however, likely that the average value is actually somewhat higher since the reaction is only linear with time within the first 10 to 20 minutes. In 15 cases of congenital

*It should be noted that the absolute density difference brought about by addition of UDPG dehydrogenase to the control is about 0.400 (Fig. 1), whereas the specimen illustrated in Fig. 2 showed a density difference of 0.300 although the same amount of UDPG was added. The purity of commercial UDPG samples seems to vary somewhat from batch to batch. Moreover, different hemolysates seem to contain different amounts of factors which influence the density increase. These fluctuations, the nature of which are not clear, do not disturb the assay at all. In all the cases of galactosemia, for instance, the difference between the density increases in the two samples is within 10 per cent.

galactosemia we have found that this enzyme is either absent or present in amounts which are so low that it cannot be detected with this method.[3] (Table II.) The method permits the detection of activities as low as 0.1 μM per Gm. hemoglobin and if the incubation time is extended, activities down to 0.05 μM can be detected. In all the cases of congenital galactosemia, regardless of age, sex, or diet, the activity was found to be below 0.05 μM and hence below the detectable level. The other enzymes involved in UDPG metabolism, such as UDPGal-4-epimerase or UDPG pyrophosphorylase, have been shown to be present in normal amounts in congenital galactosemia.[3, 6] Usually the amounts of UDPG pyrophosphorylase are so high that all, or more than 75 per cent, of the UDPG is consumed under the conditions described.

TABLE II. ACTIVITY OF PGAL URIDYL TRANSFERASE AND UDPG PYROPHOSPHORYLASE IN HEMOLYSATES OF RED BLOOD CELLS FROM NORMAL AND GALACTOSEMIC SUBJECTS
(Rates expressed as uridyl transfer, micromoles per Gm. hemoglobin per hr., 37° C.)

PATIENT	PGal TRANSFERASE	UDPG PYROPHOSPHORYLASE
Family O. D.:		
Father, 33 yr., normal	3.36	3.75
Mother, 28 yr., normal	2.19	
Daughter, 6 yr., normal	2.01	3.75
Son, 4 yr., galactosemic	<0.06	7.50
Daughter, 2 yr., 6 mo., galactosemic	<0.06	7.20
Son, 3 mo., galactosemic	<0.06	5.25
Grandfather, 66 yr., milk "intolerance"	2.25	4.50
Family T.:		
Father, 35 yr., normal	1.89	
Mother, 30 yr., normal	1.71	4.20
Son, 6 yr., galactosemic	<0.06	1.75
Family W.:		
Son, 5 yr., normal	2.40	3.48
Daughter, 2 yr., galactosemic	<0.06	3.75
T. B., 24 yr., M., galactosemic	<0.06	5.25
R. N., 2 yr., M., galactosemic	<0.06	4.80
R. T., 3 yr., M., galactosemic	<0.06	6.60

Example.—An example of an analysis of filtrate from a hemolysate of a nongalactosemic hepatitis patient is illustrated in Fig. 1; curves are shown for UDPG incubation with hemolysate with and without further addition of Gal-1-P. DPN is indicator and UDPG dehydrogenase indicator enzyme. In this case close to 90 per cent of the UDPG added was converted into UDPGal.

Fig. 2 shows an analysis of hemolysate from a galactosemic patient. It can be seen that in this case there is no consumption of UDPG even in the presence of a large excess of galactose-1-phosphate.

As indicated by Fig. 1, the consumption of UDPG proceeds rapidly during the incubation and the value found after 30 minutes incubation usually corresponds to a consumption of UDPG which amounts to at least 75 per cent of the amount added. The reaction rate is considerably higher during the first part of the reaction when only a relatively small fraction of UDPG has been consumed or if higher concentrations of UDPG are employed. Curiously enough even a moderate increase in the UDPG concentration brings about a decrease in the rate of transfer; this procedure seems, therefore, undesirable.

Shorter incubation periods and employment of less hemolysate should, however, provide appropriate conditions for studying rates which are strictly linear with time and hence maximal. It should be emphasized that the aim of the

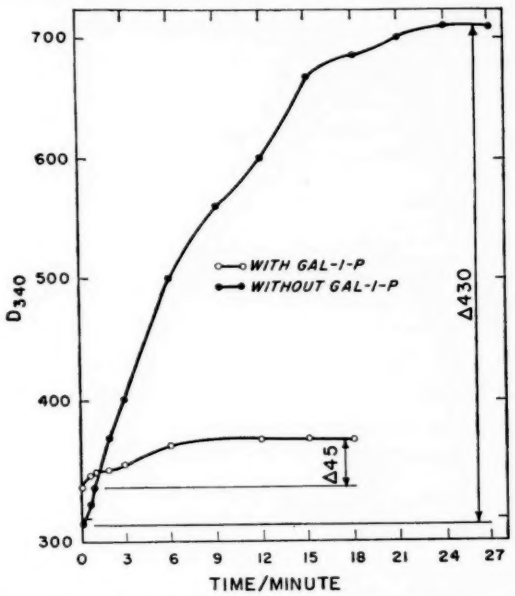

Fig. 1.—Analysis of filtrate from a nongalactosemic subject.

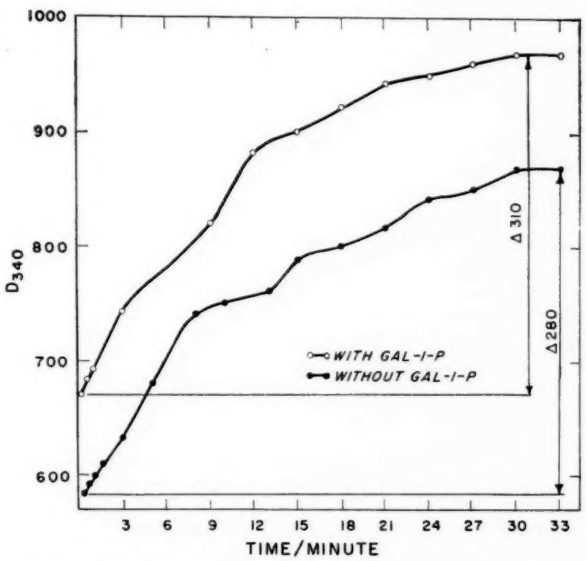

Fig. 2.—Analysis of filtrate from a galactosemic subject.

assay described in this article is not to study detailed kinetics, but to provide a field test for screening individuals with congenital galactosemia from other individuals.

These considerations are brought up here since galactose tolerance tests on parents of galactosemic children have revealed that one of the parents usually shows a somewhat lowered tolerance.[12] Thus far we have been unable to detect any strikingly lowered activity of the PGal uridyl transferase with our test. If such a decrease should be present as a trait, it must be of only moderate proportions. It would, therefore, only be detected under conditions which permit a study of maximum rates. Hence, in order to study traits in galactosemia by means of the enzymatic assay, it is mandatory to design the assay in such a way that initial rates can be recorded.

It should be noted that the titer has not been found to be altered in patients suffering from milk allergy or from various kinds of hepatitis, nor was it lowered in two cases of pregnancy observed shortly before term. The latter two conditions have been found to be associated with abnormal galactose tolerance curves.[13, 14] It may be inadvisable to assay blood after injection of large amounts of nicotinamide or nicotinic acid. It is known that nicotinamide and especially nicotinic acid plus glutamine, bring about an increase in the DPN content of erythrocytes,[15] which in turn could cause undesirable side reactions.

The assay can be run quite satisfactorily on cord blood of the newborn[4] and this would seem to be the most feasible procedure for diagnosis of galactosemia in cases where familial history would indicate that it might be expected in the new offspring. The procedure offers advantages over the drawing of a blood sample from the infant after birth in certain cases where tendency to bleeding is a very dominant feature.[16] However, it should be borne in mind that admixture of small amounts of maternal blood would, if the latter is normal, give rise to a detectable amount of the enzyme.

SUMMARY

Details of an enzymatic assay for galactose-1-phosphate uridyl transferase in blood are described; the assay is based on the consumption of uridinediphospho-glucose in the presence of excess galactose-1-phosphate. The absence of this enzyme in blood is characteristic of congenital galactosemia, and the test serves to diagnose cases of this disorder.

For valuable technical assistance thanks are due to Mr. Elwood Bynum and to Miss Bodil Waage-Jensen, trainee under the American Scandinavian Foundation through a grant-in-aid made available to one of us (H. M. K.) by the Eli Lilly Laboratories.

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A SPECIFIC ENZYMATIC ASSAY FOR THE DIAGNOSIS OF CONGENITAL GALACTOSEMIA

II. The Combined Test With 4-Epimerase

Elizabeth S. Maxwell, Sc.D., Herman M. Kalckar, M.D., Ph.D., and Elward Bynum, B.S.
Bethesda, Md.

THE conversion of the galactose structure into that of glucose in the living cell[1] proceeds according to the following equation: UDPGal* ⇌ UDPG. The enzyme catalyzing this reaction is called UDPGal-4-epimerase[2] and is abbreviated 4-epimerase. (This enzyme has previously been called galacto-waldenase.) 4-Epimerase from mammalian sources requires DPN as a cofactor.[3] As mentioned in the previous article,[4] hemolysates from the human erythrocytes do not catalyze the conversion of UDPGal to UDPG unless DPN is added. This applies to hemolysates from normal as well as from galactosemic individuals. Hence, UDPGal formed by the enzymatic reaction between UDPG and Gal-1-P, catalyzed by the enzyme Gal-1-P uridyl transferase, will accumulate. This makes it feasible to estimate the extent of the reaction by the consumption of UDPG brought about by addition of Gal-1-P. This assay is called the consumption test.[4] It would be desirable to reinforce the reproducibility and specificity of the diagnostic assay for congenital galactosemia by combining the estimation of UDPG consumption with an additional estimation of UDPGal formation brought about by the addition of Gal-1-P. This can be done by adding purified 4-epimerase to the filtrate at the termination of the UDPG estimation. Recent work[5] has made it possible to fractionate and isolate 4-epimerase in sufficiently large amounts to make it feasible, within the near future, to have 4-epimerase commercially available.

METHODS

Stock Solutions.—Stock solutions are identical with those described in the first paper of this series[4] with the addition of UDPGal-4-epimerase. The preparation of this enzyme will be described in a paper to be published shortly.†[5]

Special Equipment.—See paper I of this series, p. 469, this issue.[4]

Procedure.—Preparation of blood sample, estimation of hemoglobin, incubation of hemolysates, and deproteinization of the latter are the same as already described.[4]

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Md.

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*The following abbreviations are used: UDPGal for uridinediphosphogalactose, UDPG for uridinediphosphoglucose, DPN for diphosphopyridine nucleotide, Gal-1-P for α-galactose-1-phosphate.

†The fractionation of acetone powder from liver for UDPGal-4-epimerase which involves isoelectric precipitation and fractionation with acetone and ammonium sulphate can now be performed with great facility and in a reproducible way. The fractionated enzyme can be lyophilized and kept in this state for months without serious loss of activity. A reprint of the purification procedure can be obtained on request by communication with the authors of the present article.

The determination of the UDPG in the filtrate is also performed exactly as described in the first paper of this series.[4] However, when the density increase after addition of UDPG dehydrogenase has leveled off, 4-epimerase in the amount of 20 μl (containing 100 to 300 units*) is added first to the control cuvette and subsequently to the cuvette containing galactose-1-phosphate. The third cuvette containing inorganic pyrophosphate is not assayed with this enzyme. After the addition of 4-epimerase, the assay mixture is mixed by placing a piece of parafilm on the top of the silica cuvette and inverting it rapidly several times. Readings at 340 mμ are obtained at 30, 60, and 120 seconds after the addition of the enzyme. In order to check for changes in turbidity, the density at 400 mμ is read at 2½ minutes and again at the termination of the assay. The density at 340 mμ is read at various intervals (i.e., 5, 10, 15, and 20 minutes). The density at this wave length is followed until the increase has leveled off. This usually requires 40 to 60 minutes. The same routine described for the first cuvette is carried out for the second, which can be started 4 to 6 minutes after the first. The density increase at 340 mμ in the control should be zero or negligible (occasionally a slight decrease appears in the experimental sample). The number of micromoles of UDPGal formed by interaction of Gal-1-P with UDPG should be equivalent to the amount of UDPG consumed (see Fig. 1). The calculation of activity is carried out as described in paper I.[4] Inasmuch as the density increase due to addition of 4-epimerase often proceeds at a slower rate than that caused by addition of UDPG dehydrogenase, the end point may not be as sharp in the second assay period as in the first. Hence, the accuracy of the determination obtained by the 4-epimerase may not be as high as that obtained in the consumption test. If the accuracy of the consumption test is estimated to about ± 10 per cent, that of the 4-epimerase test may be estimated to the order of ± 15 to 20 per cent.

RESULTS

The results obtained using the test described with hemolysates from three normal and one galactosemic adult are shown in Table I. The Δ D/$_{340}$ in the

TABLE I. ANALYSIS OF FILTRATES BY THE CONSUMPTION TEST AND BY THE COMBINED TEST WITH 4-EPIMERASE

SUBJECT	Δ D/340 WITH DEHYDROGENASE		Δ D/340 WITH 4-EPIMERASE	
	WITH GAL-1-P	WITHOUT GAL-1-P	WITH GAL-1-P	WITHOUT GAL-1-P
1. Normal adult	0.025	0.405	0.340	−0.010
2. Normal adult	0.025	0.395	0.350	+0.010
3. Normal adult	0.130	0.360	0.250	+0.020
4. Galactosemic adult	0.305	0.300	0	0

first two columns of the table is due to reduction of DPN by UDPG and the specific dehydrogenase. It is, therefore, a measure of UDPG remaining after incubation of hemolysates and UDPG with or without the addition of Gal-1-P. In the case of normal individuals there is less UDPG remaining, and hence less Δ D/$_{340}$, when Gal-1-P is present during the incubation with hemolysate than when Gal-1-P is not added. This is due to the presence in the hemolysate of the enzyme Gal-1-P uridyl transferase, which forms UDPGal from UDPG and Gal-1-P. Since this enzyme is missing in hemolysates from galactosemic individuals, there is no disappearance of UDPG upon incubation with Gal-1-P. The amount of UDPG remaining, and hence the Δ D/$_{340}$, is the same after

*One unit is a density increase at 340 mμ of 0.001 per minute in 0.5 ml. with optimal substrate concentration.[5] Since the conditions in filtrates of hemolysate are not optimal, the density increase in the present assay will be 0.020 to 0.040 per minute.

incubation with or without Gal-1-P. The Δ $D/_{340}$ in the last two columns is due to DPN reduced in the same cuvettes after conversion of UDPGal to UDPG by the 4-epimerase. It is, therefore, a measure of UDPGal formed by the enzyme Gal-1-P uridyl transferase upon incubation of hemolysates with UDPG and Gal-1-P. Since hemolysates from normal individuals contain this enzyme, there is UDPGal formed, and hence a high Δ $D/_{340}$, when Gal-1-P is present during the incubation. When Gal-1-P is not added, there is no UDPGal formed and hence no Δ $D/_{340}$. In the case of galactosemic individuals there is no UDPGal formed, and hence no Δ $D/_{340}$ even when Gal-1-P is added. Figs. 1 and 2 are graphic representations of two of the same determinations.

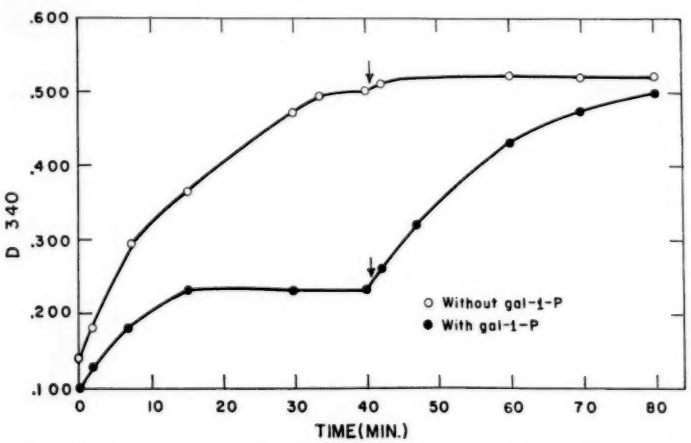

Fig. 1.—Analysis of filtrate of normal adult. UDPG dehydrogenase added at 0 minute; 4-epimerase added at 40-minute point (indicated by arrow).

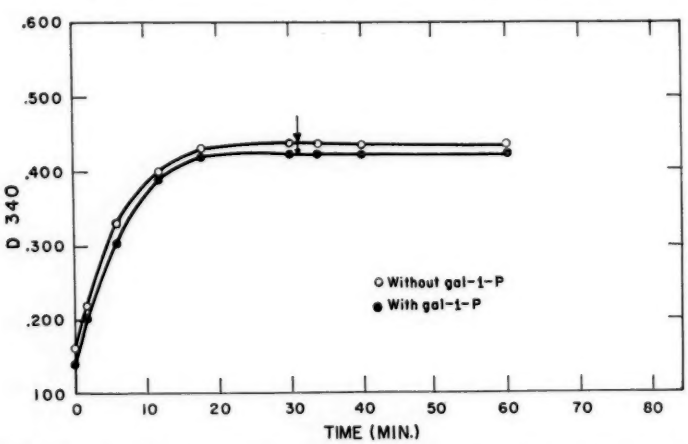

Fig. 2.—Analysis of filtrate in galactosemic adult. UDPG dehydrogenase added at 0 minute; 4-epimerase added at 30-minute point (indicated by arrow).

SUMMARY

The first paper of this series described a specific enzymatic assay for the diagnosis of congenital galactosemia. This test was based on the determination

of the amount of uridinediphosphoglucose utilized when this uridine nucleotide is incubated with red blood cell hemolysates and galactose-1-phosphate. The present paper extends the assay to include not only a measure of uridinediphosphoglucose utilized but also a measure of uridinediphosphogalactose formed during the incubation.

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ASPIRATION OF BONE MARROW FROM THE ANTERIOR SUPERIOR ILIAC SPINE

R. J. LEFFLER, M.D.
KNOXVILLE, TENN.

THE purpose of this article is to describe briefly an apparently new method of bone marrow aspiration which has been highly satisfactory in my hands for the past eight years.

METHOD

The site of puncture is the anterior superior iliac spine. It is located with fingers as an oval protuberance (Fig. 1). Following preparation of the skin and after local anesthesia has been effected, the puncture needle is lodged securely in the periosteum in the middle of this protuberance. The patient should be lying flat, preferably on a firm surface. With the pelvis in this position the needle is pressed into the ileum pointing slightly cephalad (Fig. 2).

Fig. 1.

Fig. 2.

Fig. 1.—The oval protuberance of the superior iliac spine is located between the thumbs and forefingers.

Fig. 2.—The needle is lodged in the middle of the superior iliac spine pointing slightly cephalad.

In children this method allows for better control of the puncture site. An assistant places one hand on the opposite hip and the other arm on the thighs. A second assistant holds the arms. The operator places the hypothenar eminences of both hands so as to

From the Laboratory Department of East Tennessee Baptist Hospital, Knoxville, Tenn.
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exert pressure for control on the hip. The needle can be lodged with the thumb and forefinger. Pressure is then applied with one hand while control of the hip is maintained with the other.

DISCUSSION

The superior iliac spinal puncture has been easier than sternal puncture in my hands. This has been in a large part due to increased cooperation of the patient. There is considerable apprehension by the patient when a large needle is introduced into his chest just over the heart. This apprehension is often shared by the operator, particularly when he seldom performs bone marrow aspiration. While the incidence of injury of underlying structures has not been great in sternal puncture, it is nevertheless a possibility.[1]

Fig. 3.—Longitudinal section of the superior iliac spine showing thinner compact bone at the site of puncture, indicated by arrow.

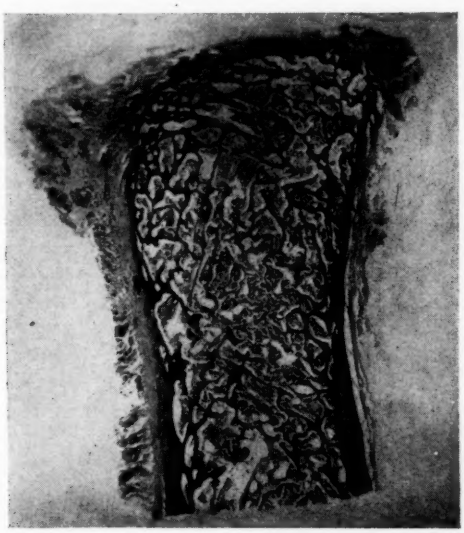

Fig. 4.—Cross section of the superior iliac spine at the site of puncture showing the compact bone of the lateral wall of the ilium.

The iliac spine is a readily accessible area. Its movement relative to the puncture can easily be controlled by the technique described. This is particularly important in obtaining bone marrow from children. Firm pressure may

be applied to the needle when lodged in the iliac spine without trepidation. Since the bone of the spine is relatively porous, it will not allow the needle to slip off into the soft tissues, once it has been correctly lodged. In case the needle should suddenly puncture through an easily yielding cortex, as occasionally happens in bone which has been altered by hyperplastic bone marrow, the momentum can carry the needle point to only a dense, oblique, iliac wall cortex.

Microscopic study of the superior iliac spinal bone structure indicates that it is particularly favorable for marrow aspiration. The cortical bone density over the spine at the site of puncture is less than over the iliac crest (Fig. 3) and is considerably less as compared to the lateral wall of the ilium (Fig. 4). It is anatomically advantageous to lodge the needle in the middle of the iliac spine and to introduce it with a 15 degree cephalad direction as described, but considerable margin of deviation from the 15 degree angle will be satisfactory. When the needle is lodged exactly in the center of the iliac spine, even extreme angles will allow the needle to enter the marrow space.

The iliac bone marrow has been satisfactory for diagnostic purposes. Duplicate studies, when done with sternal marrow specimens, have indicated no variations. This has also been the experience of others.[2] Occasional dilution of specimens was encountered, but inadequate or unsatisfactory specimens were confined to cases of bone marrow hypoplasia and similar conditions, which were subsequently demonstrated by repeated aspiration at several sites, and marrow biopsy.

SUMMARY

Aspiration of bone marrow from the anterior superior iliac spine is described and discussed as having advantages over the methods generally in use.

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A FLUOROMETRIC METHOD FOR TOTAL SERUM CHOLESTEROL

D. B. McDougal, Jr., M.D.,* and H. S. Farmer
St. Louis, Mo.

A METHOD for serum cholesterol was needed for the rapid analysis of the small amounts of blood available from the tip of a rat's tail. A simple method was developed which requires 4 μl of serum or less. Aside from its high sensitivity, the method offers certain advantages in ease of manipulation over methods currently in use in clinical laboratories.[1-3] It was thought, therefore, that the method might be of interest to those concerned with clinical estimation of cholesterol. The procedure is based on a fluorometric method previously published.[4]

MATERIALS AND EQUIPMENT

Acetic acid, acetic anhydride, and sulfuric acid meeting A.C.S. specifications for analytic reagents may be used without further purification. 1,1,2-Trichloroethane† is purified as follows[4]: it is distilled, the fraction boiling at 111° to 113° C. is collected, washed 4 times with 0.1 volume of concentrated sulfuric acid, twice with water, and dried over anhydrous sodium sulfate. Cholesterol standard solutions (100 to 400 mg. per cent) are prepared in trichloroethane.

Lang-Levy constriction pipettes[5, 6] are used for volumes less than 1 ml. For concentrated sulfuric acid, because of the high viscosity, the tip and constriction need to be rather wide.

Fluorometer tubes are selected from 3 ml. (10 × 75 mm.) Pyrex test tubes. They are cleaned by rinsing twice with a detergent solution (Dreft, Procter and Gamble; other detergents may be as good or better but have not been tested) and 4 times with distilled water.

PROCEDURE

Four microliters of serum are added to 40 μl of a 3:2 mixture (V/V) of glacial acetic acid and trichloroethane in 10 × 75 mm. tubes. A precipitate forms, but will dissolve with thorough mixing (if care is not taken to dissolve the precipitate at this point the results will be erratic and low). To the clear solution, 1 ml. of a freshly prepared mixture of trichloroethane and acetic anhydride 5:1 (V/V) is added by syringe pipette‡ and mixed. 40 μl of concentrated sulfuric acid are then added with immediate and thorough mixing.§

From the Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo.

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*Fellow of the National Foundation for Infantile Paralysis.

†No. T851, Distillation Products Industries, Rochester, N. Y.

‡Mr. Herman Ruf, Northern Tool and Instrument Co., 162-38 Pidgeon Meadow Road, Flushing 58, Long Island, N. Y.

§Rapid, efficient mixing is easily obtained by "buzzing,"[7] which eliminates the need for the introduction of any implement into the solution. This may be accomplished with simple equipment in use in this laboratory for many years. It is believed that buzzing would save time and equipment in many laboratory operations and merits a brief description. Tubes of 2 to 10 mm. diameter are held firmly against a steel cone mounted on the shaft of a small motor (1/25 H.P., 5,000 r.p.m., Type NSE-11 Bodine Electric Company, Chicago, No. 2168, 1950). The cone, 2 in. in over-all length, is turned from a ½ in. steel rod. The taper occupies 1½ in. of the over-all length. A 5/16 in. hole ½ in. deep is drilled in the large end at an angle of about 2° with the axis. (The tip of the cone is, therefore, about 1/16 in. off center whereas the base is nearly on center.) The speed of the motor is governed by a variable transformer (Type 20 Powerstat, Superior Electric Company, Bristol, Conn.), the smaller the tube the higher the speed required. Tubes of 10 to 18 mm. diameter may be stirred equally well with a larger motor (¼ H.P., 1,750 r.p.m.) fitted with a slightly bent steel rod. No loss of contents occurs provided the tube is no more than one-third to two-fifths full.

The mixture is centrifuged, and the clear supernatant solution is poured into fluorometer tubes. The samples are read using Corning filters Nos. 4010 and 5120 for the primary, isolating the 546 mμ mercury line, and No. 2424 (a cut-off filter with 37 per cent transmittance at 590 mμ) for the secondary. Solutions of 0.01 to 0.1 mg. per cent safranine O* or rhodamine B† in 0.01 N HCl may be used in the reference tube to set the fluorometer. At least forty minutes are allowed to elapse between the addition of sulfuric acid and reading. Thereafter time is not critical, but after several hours two phases separate and readings are no longer accurate.

To the blanks, 4 μl of water are added instead of serum. Standards are prepared by adding 4 μl of water and 5 to 40 μg of cholesterol in 5 to 10 μl of trichloroethane to the acetic acid–trichloroethane mixture. At final concentrations of cholesterol above 5 μg per ml., fluorescence is not proportional to the concentration and calculation may be made from a standard curve.

If serum is not limited and pipetting 4 μl seems difficult, a larger volume of serum may be diluted with 10 volumes of the acetic acid–trichloroethane mixture. A 40 μl aliquot of this may be added to 1 ml. of the trichloroethane–acetic anhydride mixture, and the analysis completed as described.

TABLE I. CHOLESTEROL RECOVERY BY PROPOSED METHOD

SERUM CHOLESTEROL		CHOLESTEROL ADDED	CHOLESTEROL RECOVERED	
MG. %	μG IN SAMPLE	(μG)	(μG)	(%)
214	9.05	6.12	5.88	96
			5.88	96
			5.45	89
			5.55	91
198	8.36	6.12	5.47	89
			5.76	94
			5.76	94
			5.97	98
163	6.88	6.12	5.97	98
			5.77	94
			6.45	105
			6.45	105
266	11.23	6.12	5.35	88
			5.54	90
			5.77	94
			5.54	91
269	11.36	15.35	15.84	103
			15.72	102
			14.86	97
			15.09	98
507	21.40	15.35	14.37	94
			13.95	91
			14.30	93
			14.47	94
338	14.23	15.35	14.60	95
			15.79	103
			14.70	96
			15.02	98
Average				96

COMMENTS ON THE METHOD

The initial dilution of serum with the acetic acid–trichloroethan mixture permits subsequent addition of a large volume of trichloroethane–acetic anhydride without trapping cholesterol in the precipitate. Without this preliminary

*National Aniline Division, New York, N. Y.
†Distillation Products Industries, Rochester, N. Y.

step, the results are invariably low. When 5 to 17 volumes of acetic acid–trichloroethane are mixed with 1 volume of serum a clear solution results. With less than 5 or more than 17 volumes the mixture is cloudy.

The concentration of sulfuric acid used is optimal. With lower concentrations, development of fluorescence is slower and incomplete. With higher concentrations, two liquid phases are present.

After the addition of sulfuric acid the precipitate may stick to the walls of the tube above the solvent level. This has no effect on the precision of the results.

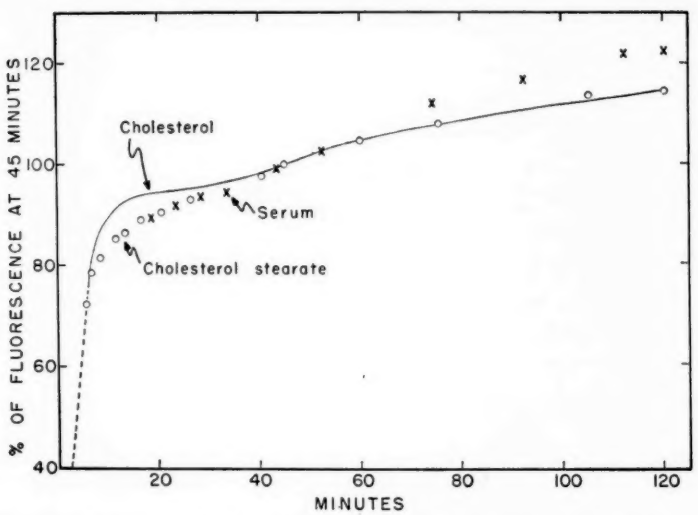

Fig. 1.—Development of fluorescence with time for cholesterol, cholesterol stearate, and serum extract. The tubes contained 6.88 μl of cholesterol solution (4 mM, 155 mg. per cent) or cholesterol stearate (4 mM) or 4.22 μl of serum (270 mg. per cent) in a final volume of 1.09 ml. Water (4 μl) was added to cholesterol and cholesterol stearate tubes. The actual readings at 45 minutes were cholesterol 75.0, cholesterol stearate 75.6, and serum 90.7 (interpolated).

RESULTS

After the initial rapid development of fluorescence, there is a slow rise (about 20 per cent per hour, Fig. 1). However, since the percentage change in fluorescence with time is about the same for the samples and the standards, this rise does not affect the results if the tubes are read in the order in which sulfuric acid was added, and the readings may be made at leisure.

There is little difference between the fluorescence developed from cholesterol and cholesterol stearate on an equimolar basis, and what difference there is disappears after 30 minutes (Fig. 1).

Since substances other than cholesterol will interfere with this method only to the extent that they do with the method of Albers and Lowry,[4] it is calculated that the hormonal steroids and bile salts present in blood contribute less than 3 mg. per cent to the final apparent cholesterol level.

Recovery experiments were performed, and the results are given in Table I. Recovery of added cholesterol averaged 96 per cent. It is uncertain whether recovery by other methods may not also be incomplete since it is difficult in those methods to introduce cholesterol in a valid manner.

The results obtained with the new method were compared with those obtained by the method now in use in Barnes Hospital*[1] and with those obtained by the method of Albers and Lowry.[4] The correspondence was good (Table II).

TABLE II. CHOLESTEROL VALUES OBTAINED BY THREE DIFFERENT METHODS

SERUM NUMBER	SAIFER AND KAEMMERER[1] (MG. PER CENT)	ALBERS AND LOWRY[4] (MG. PER CENT)	PRESENT METHOD (MG. PER CENT)
1	217	206	209
2	157	187	187
3	158	154	160
4	272	250	268
5	580	531	522
6	333	323	333
7	245	254	262
Average	280	272	277

DISCUSSION

The present method appears to be simpler than methods now in use for serum cholesterol. Thirty to sixty samples may be run in three hours or less, and the samples are $\frac{1}{25}$ to $\frac{1}{125}$ the size of those required by other methods. No attempt has been made to develop maximum sensitivity, but a ten to twenty fold increase could doubtless be obtained merely by a corresponding reduction in the volumes used.

The method has been used for repeated determinations on the sera of rats with satisfactory results. It has also been successfully applied to rat and human liver homogenates.†

SUMMARY

A simple, rapid, accurate fluorometric method for the assay of total serum cholesterol is described requiring as little as 4 μl of serum. Sixty analyses may be run in less than three hours.

Grateful acknowledgment is made to Drs. O. H. Lowry and E. Robins for help and encouragement.

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*We are indebted to Dr. H. M. Perry of the Department of Medicine for these determinations.

†Personal communication, Dr. H. B. Burch.

A RAPID METHOD FOR PREPARATION OF PLASMA PROTEINS FOR RADIOASSAY

JOSEPH KATZ, PH.D., ALVIN L. SELLERS, M.D., AND JESSIE MARMORSTON, M.D.
LOS ANGELES, CALIF.
WITH THE TECHNICAL ASSISTANCE OF SHIRLEY RAUCH, B.A.

THE use of plasma proteins labeled with radioisotopes in metabolic studies is quite extensive. For beta ray assay, two general methods of sample preparation have been used. In one, the purified fractions are combusted to carbonate or sulfate.[1] In the other, the proteins are converted to fine dry powders.[2] Both methods are slow and tedious, the first requires combustion and the second requires repeated washings and centrifugations.

A simple and rapid method is described here for preparing plasma protein for beta ray assay. Protein is flocculated by hot trichloroacetic acid and the precipitate plated on filter paper. Another procedure using protein mounting on filter paper has also been described by Armstrong and associates.[3]

MATERIALS AND METHODS

Reagents.—Three and ten per cent solutions of trichloroacetic acid, acetone, and acetone-ethyl ether (1:1) are employed.

Materials.—Whatman No. 540 filter paper. This is available in diameters of 2.1, 2.4, and 3.7 cm. All these sizes are used in this laboratory and the choice depends on the amount of protein (see below) and the type of Geiger tubes available. Most commercially available thin mica window tubes have an internal diameter of about one inch. The diameter of the protein precipitate is 4 to 6 mm. smaller than that of the paper; therefore, with these tubes, only the two smaller sizes of paper can be used. With metalized plastic window tubes of a larger internal diameter, use of the 3.7 cm. paper is advantageous and permits the use of larger amounts of protein.*

The filtering device for use with 2.4 cm. paper is shown in Fig. 1.† A coarse sinter and well-ground surfaces are important. For efficient operation two such filters are used simultaneously.

Procedure.—An aliquot of plasma, serum, or protein solution containing 5 to 60 mg. of protein (see below) in a 20 × 150 ml. tube is diluted with 0.9 per cent sodium chloride solution to 5 ml. Ten milliliters of 10 per cent trichloroacetic acid are added and the tubes heated for 10 minutes in a gently boiling water bath.

To assay plasma albumin, aliquots of plasma in plastic tubes are diluted to 1.0 ml. with 0.9 per cent sodium chloride; 5 ml. of 33 per cent ammonium sulfate is then added. The

From the Institute for Medical Research, Cedars of Lebanon Hospital, and the Department of Medicine, University of Southern California School of Medicine, Los Angeles.

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*Covered plastic holders in book form containing 20 to 25 papers facilitate greatly the handling and storage of the samples. These are available from Research Specialties Co., 2005 Hopkins Avenue, Berkeley, Calif.

†The filters can be ordered from California Glassblowing Co., 35 South Raymond Avenue, Pasadena, Calif.

tubes are centrifuged at high speed and the supernatant, containing the albumin poured into 10 ml. of 10 per cent trichloroacetic acid and processed as described above for total plasma proteins.

The filter papers are put on the filter and wet with water, the suction turned on, and the funnel clamped onto the base. The tubes are capped with parafilm and the flocculated precipitate is dispersed by vigorous shaking or by a few strokes of Teflon homogenizer pestle and poured on the filter. The precipitate is washed several times with 3 per cent trichloroacetic acid, then with acetone, and finally with acetone-ether. The papers are dried for a few minutes by suction. The drying can be hastened by the use of an infrared lamp (with the paper still on the filter under suction) and requires only 2 to 3 minutes. Using two filters, 8 to 15 samples can be filtered and dried per hour.

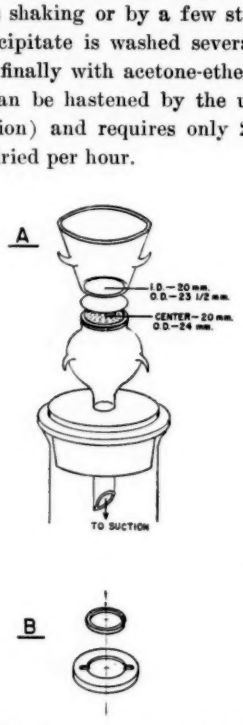

Fig. 1.—*A*, Filtering device. *B*, Adapter for radioassay.

Insufficient heating with trichloroacetic acid or insufficient washing gives a gelatinous precipitate which is hard to dry and tends to curl. Excessive heating and washing yields a powdery precipitate that adheres poorly to the paper. Properly prepared samples are white and finely granular, resembling precipitates such as $BaCO_3$. The physical character of the precipitate and the ease with which it is filtered vary with the animal species, the protein fraction, and the method of fractionation. The conditions described above apply chiefly to plasma albumins and total plasma protein. With other fractions changes in heating time and concentration of the trichloroacetic acid may be required, and preliminary trials to establish the best conditions are advisable.

The protein films are smooth and uniform up to thicknesses of about 4 to 6 mg. per square centimeter; above this range some fissuring may occur, and above 7 to 9 mg. per square centimeter the protein precipitates crack and flake. As a rule, better precipitates are obtained from albumins than from globulins. For reproducible results the maximum amount that can be plated on 2.4 cm. paper is about 30 mg. of albumin and 20 mg. of globulin. Twice this amount can be used with 3.7 cm. paper.

The weight of the precipitate is determined by weighing the papers before and after mounting. The precipitates are not hygroscopic and show no noticeable change in weight when stored in plastic holders for several days. The filter papers themselves, however, in extremes of room humidity show variations up to ± 1 per cent in weight (0.5 mg. for 2.4

cm. paper); hence, blank filter papers without precipitate should be included in each run. For reproducible counting, the papers must be kept perfectly flat. An adapter for radioassay similar to those used for $BaCO_3$ is shown in Fig. 1, *B*.

RESULTS

The procedures described here were used with I^{131}- and C^{14}-labeled proteins. The reproducibility obtained with I^{131}- labeled protein* is demonstrated in Table I. Aliquots from a number of preparations were plated out on 2.4 cm. paper to yield precipitates ranging from 3 to 30 mg. at about 1 mg. intervals.

TABLE I. EFFECT OF THICKNESS OF MOUNT ON APPARENT SPECIFIC ACTIVITY OF I^{131}-LABELED PROTEINS

EXPERIMENT NO.	SPECIES	PLASMA PROTEIN	NUMBER OF SAMPLES	WEIGHT RANGE OF SAMPLES (MG.)	RANGE OF SPECIFIC ACTIVITY (CTS./MG./MIN.)	AVERAGE SPECIFIC ACTIVITY AND STANDARD ERROR* (CTS./MG./MIN.)
1	Dog	Albumin	10	3.1-9.8	14.2-17.7	16.2 ± 1.2
			12	10.5-19.3	14.0-16.8	15.6 ± 0.8
			16	20.2-32.1	12.8-15.6	14.3 ± 0.8
2	Human	Albumin	7	3.8-9.8	84.1-92.9	89.7 ± 2.9
			9	10.3-19.2	85.2-91.7	88.1 ± 1.9
			11	20.5-31.0	77.3-86.0	80.0 ± 2.7
3	Human	Globulin	6	3.9-8.8	203-226	215.4 ± 12.6
			8	10.8-16.9	194-205	200.1 ± 1.1
4	Rat	Total plasma proteins	7	4.7-9.3	150-161	156.4 ± 2.3
			13	10.8-21.7	150-162	156.5 ± 3.8
5	Rat	Albumin	23	3.6-12.6	240-274	255.2 ± 9.3
6	Rat	Albumin	14	11.2-21.9	153-169	161.0 ± 4.3

Weight intervals from 0.5 to 2 mg. Whatman No. 540 filter paper, 2.4 cm. size, area of precipitate 3.1 sq. cm. Activity assayed with thin mica end window Geiger tube with an error of ± 3 per cent.

*$X\sqrt{\frac{\Sigma X^2}{(n-1)}}$

The minimal amount of protein that can be assayed in this procedure is limited by the precision of weighing. In Table I, the variability in the apparent specific activity of three weight ranges, 3 to 10, 10 to 20, and 20 to 30 mg. is compared. The variability is due to the common statistical counting error, errors in weighing, geometry effects, and self-adsorption. With our routine procedure, protein weights are reproducible within ± 0.2 mg. and, therefore, with precipitates lighter than about 6 mg. the main source of variability is the weighing error. This accounts for the relatively large standard error in the 3 to 10 mg. range. The better reproducibility in the 10 to 20 mg. range than in the 20 to 30 mg. range is due to greater uniformity and smoothness of the

*Commonly the gamma ray radiation of I^{131} proteins is used for assay. However, when gamma ray spectrometers or other discriminating devices are not available, or the proteins are limited in amounts and of low activity, the use of the present procedure with beta ray assay is advantageous. This, with a well type scintillator, 1 μc of I^{131} yields about 1×10^6 counts per minute with a background of at least 200 c.p.m. Using a gas flow tube with a metalized plastic window, 1 μc of I^{131} yields about 0.6 to 0.7×10^6 counts, but the background is about 20 c.p.m. Therefore, to yield a count of twice background, 4×10^{-4} μc are required for a scintillator and 0.5×10^{-4} for the Geiger tubes.

precipitates in the medium range. Indeed, in the 10 to 20 mg. range, even with a large number of samples, the total error does not greatly exceed the statistical error of counting.

The self-adsorption with the hard beta radiation of I^{131} is small and, as seen in Table I, within a 10 mg. range it is not significant. By selecting a working range of 8 to 16 mg. of protein, we have avoided self-adsorption corrections altogether.

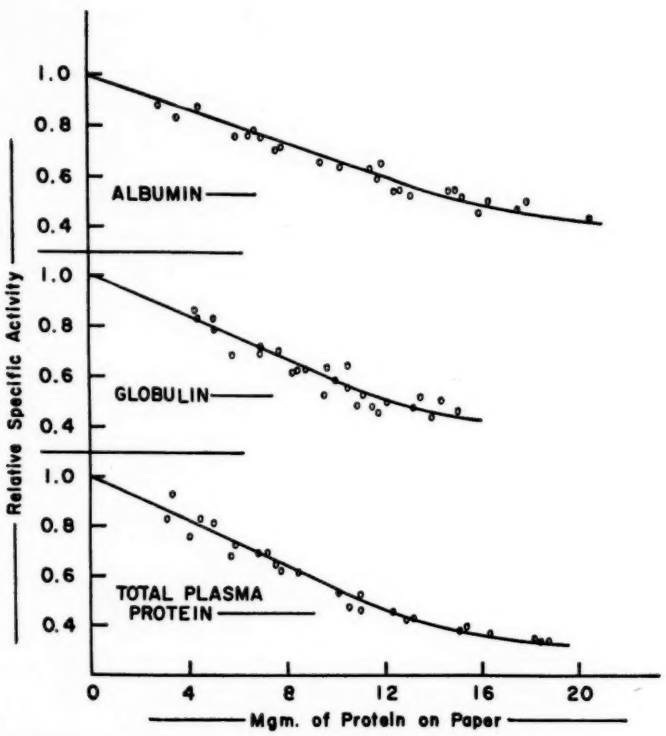

Fig. 2.—Self-absorption of C^{14}-labeled rat plasma proteins. Proteins precipitated on 2.4 cm. Whatman No. 540 filter paper. The area of precipitate is 3.1 sq. cm. Activity was assayed with a gas flow, plastic window tube. The extrapolated specific activities for 0 mass were as cts./mg./min.; 38 for total plasma protein, 51 for albumin, and 44 for globulin. Error of counting ±5 per cent.

Results obtained with C^{14}-labeled rat plasma proteins are presented in Fig. 2 where the specific activity is plotted as a function of sample weight.

The specific activities decrease linearly until 12 mg. beyond that the slopes flatten out markedly. In the linear range, the self-adsorption of the globulin and total plasma protein precipitates was about the same but larger than that of albumin. Thus, when the extrapolated specific activity at O mass is taken as 1, the observed specific activity at 10 mg. (3.2 mg. per square centimeter) was 0.55 and 0.57 for total protein and globulin, but 0.66 for albumin. The optimal weight range is from 6 to 10 mg. requiring about 0.1 to 0.2 ml. of plasma for total protein, and twice this for globulin and albumin assays.

With rats, the amount of blood that can be drawn repeatedly is limited, but when larger amounts of plasma are available the use of larger papers, thus increasing the working range, is preferable.

The method described here does not have the precision attainable when samples are combusted and assayed as $BaCO_3$; however, it has the advantage of speed and is sufficiently precise for many purposes.

SUMMARY

A simple and rapid technique for radioactivity assay of isotopically labeled plasma proteins is described. The plasma proteins are flocculated by hot trichloroacetic acid and mounted on filter paper disks. Results obtained with I^{131}- and C^{14}-labeled plasma proteins are described.

ADDENDUM*

Recently we have found glass fiber filter paper useful for mounting precipitates. This paper (made by Hurlbut Paper Co., No. X—934—Ah, distributed by Reeve, Angel and Co.) is available in 24 mm. and 37 mm. sizes. Although more retentive, it filters much faster than Whatman 540 or 542 paper; it is lighter and tends less to curl.

For assay, the papers are put in regular shallow aluminum planchets and are held in place with a flat brass washer.

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Erratum

In the article by David Platt, M.S., Howard L. Holley, M.D., and Ward Pigman, Ph.D., entitled "Changes in the Electrophoretic Behavior of Arthritic Synovial Fluid Components Following Intra-articular Steroid Therapy," which appeared in the May issue of the JOURNAL, pages 762-766, the last paragraph on page 763 should read as follows:

"Total nitrogen expressed as protein (g nitrogen × 6.25) in the active state averaged 5.0 ± 1.1 (S.D.) g per 100 ml. and nondialyzable nitrogen × 6.25 averaged 4.6 ± 1.1 g per 100 ml. Upon improvement, the average total nitrogen × 6.25 was 4.9 ± 1.6 g per 100 ml. and the average nondialyzable nitrogen × 6.25 was 4.5 ± 1.7 g per 100 ml."

*Note added in proof.

A SHORT ALBUMIN METHOD FOR THE DEMONSTRATION OF ISOHEMAGGLUTININS, PARTICULARLY INCOMPLETE Rh ANTIBODIES

MARION LEWIS AND BRUCE CHOWN, M.D.
WINNIPEG, CANADA

THE method here described, which we have used for several years, developed out of one we published in 1948.[1] It differs from those described in standard manuals[2-5] and has advantages over them: it is rapid and in our hands, using undiluted serum alone, it never misses a so-called zone Rh antibody. Furthermore it will detect weak Rh antibodies, even, in a rare case, picking up such an antibody that does not react by the saline, trypsin, or indirect Coombs methods.

No one method will detect all antibodies; the present one will pick up anti-A, B, O or H, M, S, s, P, K, k, Le^a, Le^b, the Rh antibodies and some of the rare ones. In our hands no albumin method has been successful with all anti-Fy^a antibodies; for them the indirect Coombs method is necessary.

METHODS

I. *When Several Sera Are To Be Tested With Any One Blood Sample.—*

1. Wash the cells 3 times in saline. Spin to pack, and remove the remaining saline as completely as possible with a Pasteur pipette.

2. One drop of an albumin-red-cell mixture is to be used with each serum under test (and each dilution of each serum that is to be titrated). Of 30 per cent bovine albumin,* place, to the nearest 10 drops, the number of drops so needed in a small test tube, and for every 10 drops of albumin add 1 drop of the washed, packed red cells. Mix well.

3. With a serologic pipette place 1 drop of each serum under test in a test tube 80 by 6 mm. internal diameter, followed by 1 drop of the albumin-red-cell suspension. Mix vigorously. Let stand for 10 minutes at room temperature using an automatic timer. Spin at 1,500 r.p.m. for 2 minutes. The exact timing is most important both for the original "incubation" and for the centrifuging.

4. The reaction may be read at once or may be left for reading at a later convenient time. Readings are made macroscopically by rotating the tube gently until all cells are freed from the bottom, and then gently tilting them up and down along the side of the tube. The readings are as follows:

S	one solid clump with a smooth constant edge;
S⁻	one clump that does not retain a smooth edge;
++++	2 or 3 large clumps in clear fluid; or one clump that breaks into 2 or 3 on tilting;
+++	several large clumps in clear fluid;
++	small clumps in clear fluid;
+	distinct evenly distributed fine clumps (like brick dust);
-	no clumps visible to the naked eye.

When dealing with an unknown serum all reactions are read microscopically (with a known Rh antibody, the solid to ++++ reactions would not be). Take a glass rod about 4 mm.

From the Blood Group Reference and Research Laboratory, Children's Hospital. Postal address: 735 Notre Dame Ave., Winnipeg 3, Manitoba.

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*We have found Armour's satisfactory.

in diameter by 10 cm. long, dip it in saline and place a small drop on a slide. (As many as 18 drops, 9 in each of two rows, can be placed on one slide.) Bounce the rod in a cell-albumin-serum mixture. Some cells adhere to the rod; mix these in one of the drops of saline and spread gently. With good eyesight the macroscopic and microscopic readings should agree down to a + reading. Beyond this one may read a weak + and a trace reaction microscopically, the latter no more than an uneven appearance of the cell distribution. These are recorded, respectively, as $\overline{+}$ and $\overline{\text{tr}}$, the upper bar being the macroscopic and the lower the microscopic reading.

The above is our standard method when a number, say 30 to 50, Rh-negative pregnancy bloods are being tested on one day and an average amount of serum is available. The test cells are selected to include all known antigens, at least 2 different test cells being used. Mixtures of test cells are not used because of the difficulty thereby encountered in reading very weak reactions.

II. *When Only a Small Volume of Serum Is Available.*—

A. *To test the serum against a single blood sample:*

1. Wash cells as above, removing the final saline as completely as possible.

2. Using a fine Pasteur pipette, place first in the bottom of a test tube of 50 by 5 mm. internal diameter 1 small, measured drop of serum, and then 1 drop of equal size of 30 per cent bovine albumin. (The drop used is roughly 0.02 ml. The tip of the pipette is marked with a grease pencil at the level to contain this amount.)

3. Rinse the pipette and dip the tip in the concentrated red cells. Take up a volume equal to approximately one-fifth of the volume of serum and place it in the serum-albumin mixture.

4. Mix thoroughly, flicking if necessary to get rid of any bubbles.

5. Let stand for 10 minutes at room temperature, centrifuge at 1500 r.p.m. for 1¼ minutes and read macroscopically as above. For the microscopic reading take a drop of the sediment up in a Pasteur pipette, place it in a drop of saline on a slide, and draw the mixture gently across the slide, making a pool approximately 2-3 by 10 mm.

B. *To test a serum against a large panel of cells:*

1. Add to the required amount of serum an equal voluume of 30 per cent bovine albumin. Mix thoroughly. The portion of this mixture not used at once may be stored frozen.

2. Wash red cells as above, removing all supernatant.

3. Place 2 measured drops (as above) of the serum-albumin mixture in the small test tube.

4. Add red cells as in *IIA* and proceed as there outlined.

III. *Titration of an Albumin-Active Antibody.*—Twofold serial dilutions of the serum under test are made in normal saline, and the tests are then carried out as above.

Examples of Use With High Titer and Zone Antibodies.—We have long been satisfied with this method for demonstrating the presence, specificity, and titer of albumin-active, particularly Rh, antibodies. Nevertheless, we thought it desirable to compare our method in our hands with the standard National Institutes of Health method in the hands of someone expert in the use of the latter: in this Dr. Neva Abelson kindly cooperated; we are most grateful to her.

Dr. Abelson sent us the following five sera:

No. 271. From a sterilized woman who had been stimulated to further antibody production.

No. 317. From a man with a transfusion history, whose antibody had been stimulated over a period of time.

No. 307. From a woman whose antibody was completely missed in another laboratory because of a zone.

No. 288. From a woman with a history of bearing babies with fatal or very severe erythroblastosis.

No. 323. As for No. 271.

These were all high titer sera of the sort in which a zone of greater or lesser degree may be expected to occur. We ran them by the short albumin method using R_1R_2 cells, with the results set out in Table I. Using both R_1 and R_2 cells Dr. Abelson ran them by the N.I.H. technique, and also by the present short albumin technique, which latter had been described to her in a letter. Her results are set out in Table II.

TABLE I. REACTIONS OBSERVED BY AUTHORS, USING SHORT ALBUMIN METHOD

SERUM NO.	DILUTIONS OF SERUM									
	U	2	4	8	16	32	64	128	256	512
271	S	S⁻	S	S	S	S	S⁻	+++	+	∓
317	S	S⁻	S⁻	S	S	S	S⁻	+++⁺	+⁺	tr
307	S	S	S	S	S	S	++++⁺	++	+	tr
288	S	S	S	S	S	S⁻	++++	++⁺	+⁺	=
323	S	S	S	S	S	S	S	++++	++	∓

U = Undiluted.

TABLE II. REACTIONS OBTAINED BY DR. ABELSON

SERUM NO.	CELLS	DILUTION OF SERUM										
		U	2	4	8	16	32	64	128	256	512	1024
		Using Short Albumin Method										
271	R_1	S	S	S	S	S	S	S	4	2	W1	0
	R_2	S	S	S	S	4	4	3	2	1	0	
317	R_1	S	S	S	S	S	3½	2	1½	1	W1	0
	R_2	S	S	S	S	4½	3½	2	2	1	0	
307	R_1	S	S	S	S	S	⁻S	4	3	2	1	0
	R_2	S	S	S	S	⁻S	4½	2½	1½	1	0	
288	R_1	S	S	S	S	3½	1	0				
	R_2	S	S	S	S	3	1½	1	0			
323	R_1	⁻S	⁻S	S	S	S	S	⁻S	⁻S	0		
	R_2	S	S	S	S	S	S	⁻S	4	0		
		Using N.I.H. Method										
271	R_1	⁻S	⁻S	S	S	S	S	S	S	4	2	1
	R_2	S	S	S	S	S	S	4	3	2½	W1	Tr
317	R_1	4	4½	⁻S	S	S	S	S	S	3½	3	1
	R_2	⁻S	⁻S	S	S	S	S	S	S	3	2	1
307	R_1	4½	S	S	S	S	S	S	4½	3	2	1
	R_2	S	S	S	S	S	S	⁻S	3½	2	1½	W1
288	R_1	1½	3	4	S	S	⁻S	3	2½	1½	W1	±
	R_2	1½	2	S	S	S	⁻S	3½	3	2	±	0
323	R_1	1½	4	⁻S	S	S	S	S	⁻S	3½	1½	W1
	R_2	W1	2	4	S	S	S	⁻S	3½	2	W1	±

The end points by our method in our hands are about 1 dilution less than by the N.I.H. method in Dr. Abelson's hands, while our method in her hands and in our own gives closely similar end points except for serum 323. But the end point is of relatively little importance compared with the starting point. As Dr. Abelson put it: "Nos. 288 and 323 have zones which might have been missed in an ordinary determination; the short albumin technique picked them up very well. It would certainly safeguard the ordinary laboratory technician, and eliminate most of the zones we encounter." It may be seen that, both in Dr. Abelson's hands and in our own, these 5 sera all gave solid reactions with undiluted serum, which is most important for primary demonstration of an antibody.

Since by this method zone antibodies do not zone, or do so only to a minor degree, we have little experience with the problem of zoning. The following serum was submitted to us by a laboratory of large experience. The serum was that of a group O, Rh-negative woman who had given birth recently to a baby who was jaundiced at birth and had severe anemia. The laboratory reported that the baby's cells did not react with anti-D (presumably an example of blocked D) and that no anti-Rh antibody was demonstrable in the mother's serum undiluted or diluted in albumin 1:2, 1:4, 1:8, and 1:16 and incubated with Rh-positive cells for 1 hour at 37° C. In contrast we found the titration by the short albumin method to be:

U	2	4	8	16	32	64	128
+++	S^-	S	S	S^-	++	+	=

This is the weakest reaction with undiluted serum that we have ever seen in a high titer anti-Rh antibody.

Example of Detection of a Weak Rh Antibody.—As we have stated, this method, on a rare occasion, will pick up a weak Rh antibody that does not react by the saline, trypsin, or indirect Coombs methods. Table III gives an example of such an antibody in the blood of a pregnant woman.

TABLE III

WEEK OF PREGNANCY	METHOD FOR DEMONSTRATION OF ANTIBODY* AND REACTION FOUND			
	SALINE IN CAPILLARY	10 MINUTE ALBUMIN	TRYPSIN IN CAPILLARY	INDIRECT COOMBS
33	–	+†	–	–10′
36	+	++†	+	C <1′†
39	+	+++	+	C <1′

*The antibody on each occasion and in each medium (where it reacted) was specific for D.

†C <1′ = Complete agglutination in less than 1 minute.

The baby was Rh-positive with a weak positive direct Coombs reaction and no clinical disease.

It has been our experience that no one method—saline, albumin, trypsin, or indirect Coombs—will pick up all early antibodies, and it is not our suggestion that this method should be the only one used. We mean rather to point out that it is sufficiently sensitive to replace the longer albumin methods.

SUMMARY

The standard 1 hour, 37° C. albumin method for antibody detection and titration can be advantageously replaced by a 10 minute, room temperature method. It is fast; does not miss zone antibodies missed by the standard method, and is at least as sensitive with weak antibodies as is the standard method.

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DIRECT TITRATION OF CALCIUM IN BLOOD SERUM

ROBERT L. GOLBY, M.D.,* GARY P. HILDEBRAND, M.A.,** AND
CHARLES N. REILLEY, PH.D.**
CHAPEL HILL, N. C.

THE direct titration of calcium in blood serum would save substantial amounts of time in laboratories now using the classic Clark and Collip[1] modification of the Kramer and Tisdall[2] method or the method as later modified by Tisdall.[3] A direct titration has been described by Elliott[4] using disodium ethylenediaminetetraacetate (EDTA) as a complexometric titrating agent with ammonium purpurate as an indicator. This indicator has certain disadvantages. In addition to its instability it has been the experience of the authors and other workers in these laboratories that the pink to purple color change is difficult to judge. In the presence of magnesium, as Hildebrand and Reilley[5] point out, this color change is even more gradual and indefinite. Because of these difficulties the latter authors[5] proposed the use of a new indicator (Calcon) for the titration of calcium.

Calcon, 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid, CI202, produces a blue color between pH values of 7.4 and 13.5 in aqueous solutions. Below a pH of 7.4 or above a pH of 13.5 the indicator is pink. At a pH of approximately 13, where magnesium is precipitated as the hydroxide, only calcium ions react with the indicator to form a pink-colored complex. One may then titrate the calcium directly with EDTA, the end point being detected by a color change from pink to pure blue.

The procedure proposed herein uses this indicator to detect the end point in the direct EDTA titration of calcium in serum. It is rapid, requires only one milliliter of serum, and the color change from pink to pure blue is reasonably distinct. Furthermore calcium may be determined directly in serum without prior precipitation and washing.

MATERIALS AND METHODS

Indicator Solution.—Fifty milligrams of Calcon† are dissolved in 10 ml. of methanol. This solution is stable for several weeks at room temperature.

Sodium Hydroxide 2 M.—Eighty grams of NaOH‡ are dissolved in distilled water and diluted to one liter.

EDTA Solution 0.0500 M.—Approximately 3.7 Gm. of disodium ethylenediamine tetraacetate dihydrate† are dissolved in 200 ml. of distilled water. This solution is standardized by the procedure outlined below.

Standard Calcium Solution.—Primary standard grade calcium carbonate§ is dried in an oven overnight at 110° C. Exactly 0.1001 Gm. is transferred to a 100 ml. volumetric flask

From the Department of Biochemistry and Nutrition, School of Medicine, University of North Carolina, Chapel Hill, N. C.

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*Public Health Service Research Fellow of the National Institutes of Health, Bethesda, Md. Present address: Watts Hospital, Durham, N. C.

**Department of Chemistry, University of North Carolina, Chapel Hill, N. C.

†J. T. Baker Chemical Company, Phillipsburg, N. J.

‡Reagent, Merck & Co., Inc., Rahway, N. J.

§Mallinckrodt Chemical Works, St. Louis, Mo.

with about 15 ml. of distilled water. Two milliliters of 1 M HCl are added to the flask which is then heated on a hotplate to about 60° C. to insure complete solution and evolution of carbon dioxide. The inside of the flask is rinsed down with small volumes of distilled water while the solution is still hot. After cooling to room temperature, the contents of the flask are diluted to the mark with distilled water. One milliliter of this stock standard contains 0.40 mg. calcium per milliliter. The working standard, containing 0.10 mg. calcium per 5 ml., is made by diluting 5 ml. of the stock standard to 100 ml. in a separate volumetric flask.

Standardization of EDTA Solution.—Five milliliters of the working standard (0.10 mg. calcium) are pipetted into a 50 ml. beaker, 4 ml. of 2 M MaOH are added, and the mixture is diluted to about 30 ml. with distilled water. Two drops of indicator solution are added and the solution is titrated with the 0.05 M EDTA to a pure blue end point. A "Syringe Microburet"* calibrated to deliver 0.2 μl per scale division is used. The solution is stirred continuously during the titration with an electric stirrer fitted with a small glass paddle.

The molarity of the EDTA solution is calculated by the formula:

$$\text{Molarity EDTA} = \frac{0.0025}{\text{ml. EDTA}}$$

A 5 ml. buret calibrated to 0.02 ml. may also be used, in which case the 0.05 M EDTA solution is diluted 1:50 (0.001 M) prior to standardization. The same formula is used to calculate the molarity of the EDTA.

Standardized EDTA should be stored in Pyrex or polyethylene bottles which have previously been rinsed with a hot alkaline 1 per cent EDTA solution followed by a thorough rinse with distilled water.

Determination of Calcium in Serum.—One milliliter of serum is pipetted into a 50 ml. beaker, 4 ml. of 2 M NaOH added, and the mixture diluted to about 30 ml. with distilled water. Two drops of indicator solution are added, and the solution is titrated rapidly and deliberately to a blue end point with the standard EDTA solution. Since serum may impart varying degrees of a slight yellow component to the titration, the end point may exhibit a slightly greenish tint. This color can be quickly appreciated by adding a definite excess of EDTA to several trial titrations. The true end point is reached when addition of excess EDTA causes no further color change.

The concentration of calcium in serum is calculated from the following formula:

Ml. EDTA × Molarity EDTA × 4,000 = mg. calcium per 100 ml. serum.

RESULTS

Table I shows a comparison between serum calcium values obtained by the proposed procedure and the method of Tisdall.[3] The latter procedure was used on this group of sera by the Clinical Biochemistry Laboratory of the North Carolina Memorial Hospital, Chapel Hill, N. C.

Duplicate rather than average values are presented to illustrate the reproducibility in each method. The average difference between duplicate titrations within each method itself is 0.23 mg. calcium per 100 ml. serum in the group of data shown and the average difference between the two methods is 0.25 mg. calcium per 100 ml. serum.

The results in Table II are mean values of duplicate determinations by the present method of five serum samples to which 10.0 mg. calcium per 100 ml. serum have been added. The average recovery of added calcium is 10.0 mg. per 100 ml. of serum.

A series of determinations carried out on 10 identical 1.0 ml. samples of pooled serum yielded a mean value of 10.5 with a standard deviation of ±0.15 mg. calcium per 100 ml. serum.

*Micro-Metric Instrument Co., Cleveland, Ohio.

TABLE I. COMPARISON OF PROPOSED METHOD WITH THE PROCEDURE OF TISDALL

SAMPLE NO.	CALCIUM (MG. PER 100 ML. SERUM)	
	PROPOSED METHOD	METHOD OF TISDALL[3]
1	10.4, 10.1	9.6, 10.0
2	6.6, 6.2	6.2, 6.0
3	10.0, 9.7	9.4, 9.8
4	12.3, 12.5	12.2, 12.3
5	9.3, 9.4	9.4, 9.4
6	7.2, 7.1	6.2, 6.5
7	9.5, 9.5	9.4, 9.4
8	10.2, 9.8	9.6, 10.0
9	9.9, 9.9	10.0, 10.0
10	10.3, 10.1	10.4, 10.3
11	9.3, 9.2	9.0, 9.8
12	8.7, 8.6	8.8, 8.6
13	7.2, 7.1	6.5, 6.7
14	10.5, 10.3	10.0, 10.3
15	9.9, 9.7	9.9, 9.7
16	6.4, 5.9	6.3, 6.4
17	7.4, 7.8	8.0, 8.1
18	10.0, 10.3	10.2, 9.9
19	8.9, 8.5	9.1, 9.5
20	8.5, 8.8	8.2, 8.5
21	9.8, 9.7	10.3, 10.1
22	10.8, 11.1	10.7, 10.9
23	10.4, 10.1	10.6, 10.3
24	9.2, 9.0	9.8, 9.7

TABLE II. RECOVERY OF CALCIUM ADDED TO SERUM

SAMPLE NO.	CALCIUM (MG. PER 100 ML. SERUM)		
	SERUM	SERUM, WITH 10.0 MG. CALCIUM ADDED PER 100 ML.	CALCIUM RECOVERED
1	9.4	19.4	10.0
2	12.4	22.3	9.9
3	11.0	21.1	10.1
4	7.2	17.4	10.2
5	8.9	18.9	10.0
Mean			10.0

SUMMARY

A direct titration method for the rapid determination of calcium in serum is presented. The results obtained by this method indicate a degree of accuracy and reproducibility closely approximating that of the classic permanganate titration of precipitated calcium oxalate. The average time required for duplicate titrations is less than 5 minutes.

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